MACROPHAGE PLASMA MEMBRANE RESPONSE

TO PASTEURELLA HEMOLYTICA

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CHAPTER I

INTRODUCTION

Pneumonic Pasteurellosis is a major disease problem in the beef cattle industry (1, 2). Numerous attempts to devise vaccination procedures have been for the most part unsuccessful in providing a practical and efficient method of preventing the disease (14, 16, 17-19). Extensive research into the nature of Pasteurellosis has helped to provide a better understanding of the disease and its pathogenesis, but Pasteurellosis still remains an important cause of fatalities and economic loss to feedlots and beef cattle operations in general.

The alveolar macrophage normally plays a vital role in response to respiratory infections. In Pasteurellosis it has been found that one of the etiologic agents, Pasteurella hemolytica, produces a factor which is cytotoxic and inhibitory to the phagocytic activity of the macrophage (22, 23, 24). These detrimental effects have prompted efforts to try to understand more fully the role which the alveolar macrophage plays in Pasteurella infections.

Recent investigations into the nature of the plasma membrane of phagocytic cells have applied techniques to moniter membrane potential changes in polymorphonuclear and mononuclear phagocytes. Microelectrodes have been used to determine normal membrane potential values in these cells, and the response that occurs due to various chemical agents or stimuli (32-38). Membrane potential sensitive

cyanine dyes have also been used in similar experiments, and have been found to yield results consistent with those of the microelectrode experiments (85, 90, 91).

The purpose of this study is to investigate the plasma membrane of the bovine alveolar macrophage to determine the changes it undergoes during in vitro exposure to Pasteurella hemolytica. Both microelectrode and cyanine dye techniques will be used to moniter membrane potential changes, while morphologic studies of the membrane will be made with scanning electron microscopy. Evaluation of these membrane characteristics and parameters in the alveolar macrophage will hopefully provide additional insight into the nature of the macrophage reponse to Pasteurella hemolytica.

CHAPTER II

PASTEURELLOSIS

Of all cattle diseases, 40% to 80% have been reported to involve the respiratory system (1). The economic importance of this to the beef cattle industry, particularly feedlot operations, is extremely significant. Losses due to fatalities, loss of conditioning, inefficient feed conversion, and expensive treatments run into the millions of dollars each year (2). The major cause of these losses in feedlot cattle is the shipping fever complex (3). The exact nature and etiology of shipping fever in cattle have long been sources of debate and confusion (2). Though the term shipping fever encompasses numerous etiological factors, such as stress and viral infections (1), it has also come to be used to refer specifically to the disease pneumonic pasteurellosis (4).

Bovine pneumonic pasteurellosis in its strictest sense refers to respiratory infections in cattle caused by Pasteurella multocida or Pasteurella hemolytica. Of these two organisms, P. hemolytica has been found to be the cause of most severe illness and fatalities (5). P. hemolytica is a small, rod shaped, encapsulated, Gram negative bacterium (6). These organisms produce a potent endotoxin (7), as well as an exotoxin which is still incompletely characterized (8).

Clinical Signs

Clinical signs produced by Pasteurellosis involve initial depression, loss of appetite, dry nose, and a rough hair coat. Later the animal becomes weak and progressively more depressed as purulent discharge from the eyes and nose develop. Respiration becomes difficult, and the animal continues to loose weight and conditioning (6).

Gross and Microscopic Lesions

Natural occurrence of Pneumonic Pasteurellosis produces an acute fibrinous pneumonia. Descriptions of the lesions consistently include primary involvement in the anteroventral portion of the lung, particularly in the cardiac and apical lobes, but varying degrees of lung involvement have been reported (2). Affected lobes are firm, heavy, and red to black in color. These lobes are separated from normal lobes by thick, edematous interlobular septae. Lymphatic vessels are often filled by clots of fibrin, and bronchi are hemorrhagic and also contain clots of fibrin and exudative debris (9). Reports of fibrinous pleuritis, serofibrinous pericarditis, and the presence of yellow pleural fluid are also fairly common (2).

Microscopic examination shows bronchiolitis, with plugs of exudative cellular debris and bacteria contained within the bronchioles. Alveoli are generally filled with fibrin, macrophages, and neutrophils, with the mononuclear cells being the predominate cell type present. Lymphatics and blood vessels are usually dilated and often contain fibrinous clots or are thrombosed. Multifocal areas of

coagulative necrosis are also consistent findings (2).

Lesions caused by experimentally induced infections are similar to those seen in the natural disease. These include consolidation of the apical and cardiac lobes, thickened interlobular septae, fibrinous pleuritis, fibrinous pericarditis, and the presence of yellow pleural fluid (2).

Microscopic appearance of the lesions from the experimental disease are also similar to those found after natural infections and have been evaluated at various time intervals following infection by Friend, Thomson, and Wilkie (10). Their study showed an initial accumulation of fibrin and macrophages in alveoli, with infiltration of neutrophils into alveolar spaces and bronchioles. Later, thrombosis and dilation of lymphatic vessels and areas of coagulative necrosis were also evident. Alveoli and alveolar ducts contained fibrin, neutrophils, macrophages, and occasional giant cells. Bronchioles were plugged with neutrophils and necrotic debris.

Pathogenesis

The pathogenesis of these pulmonary changes is a subject of continuing debate. A major problem in studying Pasteurellosis has been the inability to consistently reproduce the disease experimentally. Though P. hemolytica alone has been shown to experimentally produce respiratory disease (2), prior or concurrent infection with viral, Mycoplasmal, or Chlamydial agents has been found to greatly enhance the development of both the experimental and natural disease (2, 9, 11).

Pasteurella organisms have been found to be part of the normal

flora of the bovine nasal cavity (12, 13). The organisms are generally present in low concentrations in only a few animals in a group. Groups of animals which have been handled or transported, however, show an increase in both the incidence and concentration of P. hemolytica in the nasal microflora of that group (5). This supports the common view that transportation and associated stress may also play an important role in the pathogenesis of Pasteurellosis (11, 14, 15).

Pasteurella organisms have a difficult time in establishing a focus of infection on normal intact respiratory mucous membranes (14). Prior infection with other agents, however, may allow Pasteurella organisms to establish themselves in respiratory tissues. Infectious Bovine Rhinotracheitis virus (Bovine Herpesvirus 1), can cause vacuolation, detachment, and loss of ciliated epithelium in bronchi, as well as congestion, edema, focal hemorrhage, and thickening of alveolar septal tissue. This virus also has been shown to depress ciliary clearance and alveolar macrophage activity. The combination of this pulmonary tissue damage and inhibition of these normal pulmonary defense mechanisms may help to predispose the lung to a secondary Pasteurella infection (16). Myxovirus (Parainfluenza 3 virus), Mycoplasmas, and other infectious agents have also been incriminated as predisposing factors to Pasteurellosis (3, 9, 14). These agents probably function in a similar manner to that described for Bovine Herpesvirus.

Tissue damage due to Pasteurella infections has been hypothesized to occur by two mechanisms. These are the creation of pneumonia by the bacteria itself, and necrosis caused by endotoxin induced lymphatic and vascular clotting and thrombosis (9). Together these account for the lesions commonly seen in Pasteurellosis.

Vaccination Programs

Vaccination programs against Pasteurellosis have been inadequate in controlling the disease. Current research is in large part directed towards development of an effective immunizing agent, but various difficulties have been encountered with developing and testing Pasteurella vaccines (17). Two major approaches have been taken in this research. These are to vaccinate against the viruses which appear to predispose to development of Pasteurellosis, or to vaccinate with agents directed against the bacteria or its products (14). Neither method has yielded satisfactory results. Although both subcutaneous and intrabronchial routes of innoculation of Pasteurella bacterins stimulate respiratory antibodies which are recoverable in bronchial washings (18), subcutaneous injections have also been found to enhance pneumonic signs following aerosol challenge with live bacteria (17). Innoculation with aerosols made from live P. hemolytica have been found to produce the most beneficial immune response when using only Pasteurella organisms as the immunizing agent (19), but combinations of viral and bacterial agents have shown the most promise in providing protection from the disease (16).

CHAPTER III

ALVEOLAR MACROPHAGES IN PASTEURELLOSIS

Alveolar macrophages are the primary phagocytic cells of the normal lung and act as a first line of defense against many invading microorganisms (20, 21). Normally these macrophages will phagocytose foreign particles and bacteria before serious infection or inflammatory responses can occur. A primary problem in Pasteurellosis is an inadequate phagocytosis of P. hemolytica by alveolar macrophages, with subsequent proliferation of the invading organism and concurrent pulmonary damage.

Recent investigations have suggested that the macrophage may not play the major role in the immune response of the lungs to Pasteurella infections (25, 26). The reason for this inadequate macrophage response has been reportedly due to an inhibition of phagocytosis, and a cytotoxicity to alveolar macrophages caused by P. hemolytica.

Inhibition of Phagocytosis and Cytotoxicity

Benson et al. (22) were the first to report a decreased rate of phagocytosis and morphological changes indicative of cytotoxicity in alveolar macrophages exposed to P. hemolytica in vitro. Macrophages exposed to heat-killed P. hemolytica, however, showed a high rate of phagocytosis, and only minimal cytotoxic changes. Markham et al. (23) measured the rate of phagocytosis by the uptake of radiolabeled

bacteria. They found that macrophages phagocytosed Yersinia enterocolitica at a much faster rate than they did for P. hemolytica. The rate of phagocytosis of both organisms was enhanced by the presence of immune serum, but as the number of Pasteurella organisms was increased this rate decreased. They suggested that the increased uptake of opsonized bacteria initially occurring at high bacterial concentrations may in fact be detrimental to alveolar macrophages. Supporting this statement was the observation that high bacterial numbers also caused an increased detachment of cells from coverslips which was suggestive of cell death. Low concentrations of supernatents from P. hemolytica cultures were also found to inhibit phagocytosis, while higher concentrations were found to cause cytotoxicity as measured by a chromium-51 release assay technique. Maheswaran et al. (24) confirmed that P. hemolytica was poorly phagocytosed in the absence of opsonins from normal adult bovine serum or antiserum. Incubation of cultured macrophages with low (1:10) alveolar macrophage to bacteria ratios failed to produce these toxic effects; however, a 1:20 ratio caused cytotoxic changes which occurred between 30 and 90 minutes following initial exposure. The cytotoxic effect was suggested to be due to a substance released by the excessive unphagocytosed bacteria. A similar depression of phagocytosis and evidence of cytotoxicity has also been noted in polymorphonuclear leukocytes (8, 27) and blood mononuclear monocytes (28) exposed to P. hemolytica.

A recent study which evaluated the response of macrophages in vaccinated animals to P. hemolytica showed that vaccination caused an enhanced phagocytosis of these organisms by macrophages (29). The

relationship between this apparent reduction of cytotoxicity and phagocytic inhibition noted in vivo and the results obtained previously in vitro is not clear.

Nature of the Cytotoxin

The nature of the cytotoxic factor is not completely understood. In work with neutrophils, Baluyut et al. (8) found that production of the toxin by P. hemolytica is maximal during the logarithmic phase of bacterial growth but decreases during the stationary phase. Further study showed that the toxin was inactivated by trypsin, was heat labile, oxygen stable, susceptible to pH extremes, and possessed a molecular weight greater than 300,000. No endotoxin was detected to be present and this cytotoxic factor was characterized as a typical bacterial exotoxin. Studies with macrophages by Himmel et al. (30) showed the toxin to be a 150,000 dalton protein which was specific for P. hemolytica serotypes, but not Pasteurella multocida serotypes. The cross reaction of the toxin with numerous P. hemolytica serotypes suggested to them that the single antigen could be used to induce antibodies which would provide immunity against all the serotypes of P. hemolytica. Further characterization and purification of the toxin will be necessary before such a hypothesis can be adequately investigated. The molecular weights of the toxin in these studies was found to vary considerably. It is possible that the lower molecular weight toxic factor may be a subunit of the larger isolated unit. Future studies of the toxin will also be necessary to help explain these differences.

CHAPTER IV

MACROPHAGE MEMBRANE POTENTIAL - MICROELECTRODE STUDIES

The functions of macrophages are many and varied. These include enzyme secretion, chemotaxis, and phagocytosis (31). All of these functions involve events occurring at the plasma membrane. For this reason some relatively recent research has been directed towards examination of the electrical properties and ionic fluxes occurring in the macrophage membrane, and the change in these properties produced by response to various stimuli.

Initial Studies

Gallin et al. (32) used microelectrode techniques to examine the membrane properties of cultured guinea pig peritoneal macrophages. They found the cells to have an average transmembrane potential of -13.1 mV. In addition some macrophages studied showed spontaneous hyperpolarizations, usually associated with impalement of the cell. Hyperpolarizations could also be elicited by electrical or mechanical stimulation. Increasing the extracellular potassium concentration led to a change in the membrane potential predicted by the Nernst equilibrium for potassium. From this they concluded that the hyperpolarization was most likely caused by an increased membrane permeability to potassium.

In studies on mouse peritoneal macrophages similar results were obtained. Average transmembrane potentials were -26 mV, and slow hyperpolarizations occurred either spontaneously or when induced by electrical stimulation. These hyperpolarizations were found to be blocked by tetraethylammonium and were rapidly produced and sustained by valinomycin. These results provided evidence for the possible role of an increased potassium permeability as the cause of the hyperpolarizations (33).

Nature of the Membrane Potential in Macrophages

Transmembrane potential values reported for macrophages have been similar to those reported for other non-excitable cells. These values have normally ranged from -5 mV to -40 mV, with an average of between -10 mV to -15 mV (32-38). Recently, however, some cells have been found with potentials averaging -70 mV; these values are much more characteristic of potentials for excitable cells (38, 39). The reason for the predominately low membrane potential in macrophages was demonstrated in studies concerning the nature of membrane permeabilities to potassium in excitable and non-excitable cells. It was found that the resting membrane permeability to potassium was low in non-excitable cells. In excitable cells, however, the permeability of the resting membrane to potassium is much higher (40). This finding explained the relatively low resting membrane potential in non-excitable cells when compared to the higher resting membrane potential of excitable cells. The higher potentials measured in some macrophages, and subsequent results of current- voltage studies, have shown these cells to have membrane characteristics similar to those of excitable cells (38).

The significance of these findings is still unclear.

Response to Chemotactic Factors

The possible importance of these potassium permeability changes in relation to macrophage function has been examined in neutrophils. Factors which stimulate chemotaxis have also been shown to stimulate lysosomal enzyme release (41). When neutrophils were exposed to a variety of different natural and synthetically derived chemotactic factors (including complement derived factors and N-formyl methionyl phenylalanine), the presence of extracellular potassium was found to cause an increased enzyme release from these cells (42). Some of these same factors have been shown to increase membrane permeability to potassium resulting in rapid cellular potassium effluxes (44). The nature of these membrane responses in terms of potassium permeability appear to be similar during both exposure to these chemotactic factors and during membrane hyperpolarizations.

Membrane Potential Changes - Role of Calcium

The role of calcium in controlling potassium permeability has been previously shown in red blood cells (44, 45) and in secretory cells (46), and has been proven or suggested in a wide variety of excitable tissues (47, 48, 49). In the previous studies by Gallin et al. (32), they found that the spontaneous and induced hyperpolarizations of the macrophage membrane were inhibited by Mg-EGTA (a calcium chelator) and prolonged by the presence of a divalent cation ionophore (A23187). These results led them to suggest that rapid changes in the intracellular calcium concentrations may mediate the potassium induced

membrane hyperpolarizations (32).

In a recent study of macrophages induced from foreign body granulomas in rats and mice similar hyperpolarizations were demonstrated. A double microelectrode technique was used to inject calcium into the cells and measure the magnitude of the resulting hyperpolarization. The size of these calcium induced hyperpolarizations was found to be directly related to the extracellular potassium concentration which was used in the bathing media. This provided evidence for the calcium dependence of the potassium permeability changes noted during these hyperpolarizations in macrophages (36).

Similar results and conclusions were drawn from a related study by Oliveira-Castro and Dos Reis (50) involving divalent cation ionophores, variable potassium concentrations, and the calcium antagonist verapamil. In addition they suggested the possible association of these hyperpolarizations to chemotaxis, cell motility, and enzyme release by macrophages.

The role of calcium in chemotaxis was shown in a study with human neutrophils and blood monocytes. Cells exposed to chemotactic factors in the absence of calcium and magnesium showed a reduced migration which could be reversed by low doses of divalent cation ionophores. Procaine, which reduces cell membrane permeability to cations, also resulted in an inhibition of locomotion (51). Similar responses to the complement component C5a, kallikrein, and transfer factor; all potent chemotactic factors, have also been obtained. In addition, exposure to these chemotactic agents was found to result in a rapid calcium efflux, decreased calcium influx, and an intracellular shift towards a decreased cytoplasmic calcium fraction in neutrophils and monoctyes (52). Results

obtained by Boucek and Snyderman (53), however, demonstrated an increased calcium uptake by neutrophils and mononuclear cells exposed to both chemotactically active serum and the synthetic chemotactic agent N-formyl methionyl phenylalanine. This response was inhibited by lanthanum chloride. From these results they suggested that the influx of calcium into these cells may play a role in chemotaxis.

These calcium studies indicate that calcium has an important influence on the membrane potentials and chemotactic activities of phagocytic cells. The exact nature and time sequence of the calcium permeability changes and calcium induced potassium permeability changes, however, are still unclear.

Membrane Changes During Chemotaxis

The relationship of chemotactic stimulation to membrane potential changes in monocytes has since been examined by Gallin and Gallin (37). In this study exposure to endotoxin-activated serum was shown to produce large hyperpolarizations in these cells. The chemotactically active component of the serum, C5a, applied alone produced hyperpolarizations which were blocked by the presence of Mg-EGTA. Similar hyperpolarizations were produced by N-formyl methionyl phenylalanine. Simultaneous microelectrode recordings and observation of the cells showed that these membrane potential changes precede any morphological changes in the cell associated with chemotaxis, such as spreading, membrane ruffling, or pseudopod formation. These results led them to speculate on the relationship between membrane ionic movements and the morphological changes associated with chemotaxis. This speculation involved an initial increased permeability of the cell membrane in

response to the chemotactic agent and subsequent influx of calcium into the cell. This rise in intracellular calcium would increase the membrane permeability to potassium. The potassium efflux would then be responsible for the membrane hyperpolarizations recorded in this, and other studies. An identical sequence of ionic events occurring in association with cell chemotaxis has been proposed by Naccache, Showell, Becker, and Sha'afi in their work with rabbit neutrophils (43, 54).

Studies on the surface charge of polymorphonuclear leukocytes have shown that upon incubation with chemotactic factors a decrease in the surface charge occurs. These results were correlated with previous studies in the amoeba and fibroblast in which decreased surface charge was associated with cellular contraction, and increased charge with cellular expansion. The suggestion was made that these changes in surface charge were events occurring prior to cell motility (55). What, if any, association these charge differences have with induction of membrane hyperpolarizations are entirely speculative.

Membrane Changes During Lysosomal Enzyme Release

Since chemotactic factors have also been shown to stimulate lysosomal enzyme release (41) and the events of chemotaxis and exocytosis have been reported to be closely interrelated (56), calcium and membrane potential changes may possibly play a role in lysosomal enzyme release by phagocytic cells. In experiments involving addition of calcium and ionophores to neutrophils, these stimuli were found to greatly enhance lysosomal enzyme release (57). A similar study also using neutrophils obtained the same results (58). In studies with

rabbit alveolar macrophages and the divalent cation ionophore A23187 results were obtained which were identical to those seen in neutrophils (59). The enzyme secretion induced by the secretagogue A23187 has been found to decrease the surface charge of polymorphonuclear leukocytes. This same response, but to a lesser degree, was also caused by either of the chemotactic factors C5a and N-formyl methionyl phenylalanine when combined with cytochalasin B. Both responses were found to be dependent on the presence of calcium. A linear relationship was found between the decrease in surface charge and the amount of enzyme released. It was concluded that these studies showed an association between enzyme secretion (exocytosis), and the surface charge of the polymorphonuclear leukocyte membrane (60). These results indicate an important role for calcium in lysosomal enzyme release. The significance of membrane potential changes, enzyme release, and surface charges are not clear. The separation of these responses from that of chemotaxis would be difficult electrophysiologically due to the similar membrane effects of secretagogues and chemotactic agents.

Role of Microfilaments and Microtubules

Following an increase in intracellular calcium concentration which was induced by a chemotactic factor, sequestration or efflux of calcium would be expected at some point to remove this calcium from the cytoplasm (37). A selective intracellular movement of calcium is also indicated by the finding that chemotactic factors induce a submembranous accumulation of calcium in neutrophils near the edge of the cell which is closest to the chemotactic stimuli (61). In studies which indicated chemotactic factors could also cause a calcium efflux, the suggestion

was made that this decreased calcium concentration may be responsible for microtubule assembly, while local accumulations of calcium would be required for microfilament function (52). The finding that the antitubulins demecolcine and podophyllic acid ethylhydrazide will inhibit neutrophil chemotaxis, but not random movement, would indicate a substantial role for microtubules during chemotaxis (62). Activity of the microfilament system is also indicated from the result that cytochalasin B is able to inhibit both spontaneous and directed movement of neutrophils (63). In a summary of current evidence concerning microtubule and microfilament function in neutrophil activities, Becker (64) drew the following conclusions. He suggested that random movement requires microfilaments only, but chemotactic movement requires both the microfilament and microtubule systems. Phagocytosis appears to require microfilaments, while lysosomal enzyme release may be dependent upon microtubules. These suggestions point towards a major role of these systems in neutrophil function, and a corresponding importance of calcium as a mediator of both microfilaments and microtubules (65). The presence of myosin in neutrophils provides another possible role for calcium in the mediation of contractile processes associated with chemotaxis or phagocytosis (65, 66).

Membrane Changes During Phagocytosis

Changes in macrophage membrane potentials have also been recorded in actively phagocytizing cells. Rat peritoneal macrophages with average resting membrane potentials of -13 mV were exposed to latex microspheres which they are able to rapidly phagocytose. An immediate hyperpolarization of the cells occurred, reaching maximum values of

-46 mV at 15 to 30 minutes after the initial exposure, and then depolarizing back towards their initial resting values (34).

Hyperpolarizations in human polymorphonuclear leukocytes exposed to the immune complex concanavalin A, and to bovine serum albumin-anti albumin complexes have also occurred. This response was also obtained when binding of these agents to membrane receptors was inhibited by cytochalasin B. These results lead to the suggestion that membrane hyperpolarizations could be caused by a membrane receptor-ligand interaction, and that these events precede and possibly even stimulate the active process of phagocytosis (67). As with chemotaxis and exocytosis, a decrease in surface charge has been found to occur in macrophages associated with particle attachment during phagocytosis (68). The relationship of this to membrane potential changes is again uncertain.

Heterogeneity of Macrophage Membrane Potentials

Recent studies with single electrode voltage clamp techniques have indicated the presence of two stable membrane potentials for macrophages. Cultured murine peritoneal macrophages showed primarily a low membrane potential of -24.7 mV; however, a smaller number of the cells showed an average membrane potential of -70 mV. These cells were also found to exhibit different current-voltage relationships when exposed to microelectrode current pulses. The low membrane potential cells were found to have a linear current-voltage relationship for inward current pulses, while the more hyperpolarized membrane potential cells showed S-shaped current-voltage relationships. The possibility that these different characteristics may have been related to the degree

of activation of the individual cells was suggested (38).

A related study on macrophages cultured from mouse spleens showed similar results, but also found some non-linear N-shaped current-voltage responses in 10% to 20% of the cells. Two cells were even found to have stable resting membrane potentials at both the high and low membrane potential values when injected with various hyperpolarizing or depolarizing current pulses (39). These studies further indicate the complex nature of the macrophage membrane.

CHAPTER V

MACROPHAGE MEMBRANE POTENTIAL - CYANINE DYE STUDIES

The first significance of the cyanine dyes was discovered in 1873 when H. W. Vogel found that photographic plates exposed to these dyes were sensitive to wavelengths of light other than those currently used for black and white photography. The subsequent synthesis of a wide variety of cyanine dyes parallels the development of color and high speed photography (69). Today the cyanine dyes still play an important role as photographic emulsion sensitizers.

Biological Applications

The first biological application arose when the cyanine dye 3-ethyl-2-[5(3-ethyl-2-benzothiazolinylidene)-1, 3 pentadienyl] benzothiazolium iodide, also known as dithiazine, was found to be an effective anthelmintic for a wide variety of parasitic infections (70). It is still currently used in the treatment of many of these parasites. Investigations beginning in 1970 at the Marine Biological Laboratory at Woods Hole, Massachusetts indicated that certain fluorescent dyes, including the cyanines, might be of value as indicators of membrane potential changes in neurons (71). These studies culminated in a report on the use of merocyanine dye in monitering membrane potential in the squid giant axon. The suggestion was also made that the use of this

dye might have broad applications in a variety of cells and tissues (72). Since then hundreds of fluorescent dyes with many different origins have been examined for their usefulness as moniters of membrane potentials (71, 73). Of this group the dyes which are most commonly used are the merocyanines, oxonols, and cyanines (74).

Nature of Membrane Potential Sensitive Dyes

Membrane potential sensitive dyes have been classified into two main functional types, the slow dyes and the fast dyes. The fast dyes include primarily the merocyanines, but also some of the oxonols and the cyanines. These dyes are impermeant, and normally uncharged. They respond to changes in membrane potential with small, but very rapid (millisecond) fluorescence changes (75). These dyes have been employed primarily in studies of excitable tissues such as nerve and muscle preparations (74).

The slow dyes, which include most of the cyanines, are permeant, positively charged molecules which respond to membrane potential changes with slow (seconds), but relatively large fluorescence changes (75). These dyes have found a wide variety of applications in studies of membrane potential changes in suspensions of cells, organelles, and vesicles. Studies with the slow dyes have been done on red blood cells (76, 77), Ehrlich ascites tumor cells (79, 80), bacteria (81), intracellular organelles (82), membrane vesicles (83), neutrophils (85), blood platelets (84), tissue sections (86), and numerous other preparations (74).

Mechanisms of Fluorescence

The means by which these dyes respond to membrane potentials with a change in fluorescence seem to vary between the slow cyanines and the fast merocyanines (75). The mechanism for the merocyanine dyes is not completely understood. Most studies indicate that the development of local asymmetrical charges on the dye allow it to undergo potential-dependent monomer/dimer shifts which are associated with changes in fluorescence. The dye monomers appear to be membrane associated, while the dimers may be either membrane associated or free in the surrounding bathing media adjacent to the membrane. The mechanism of the slow cyanine dyes is somewhat better defined (75). Hoffman and Laris (76) were the first to show a correlation between cyanine dye fluorescence and membrane potential in their work with red blood cells. They were able to observe the dye response when changes in membrane potential were induced by varying external potassium concentrations in the presence of valinomycin, by changing external chloride concentrations, or by substituting impermeant ions for chloride ions. In each case they found a decreased dye fluorescence associated with hyperpolarization of the cell and an increase in dye fluorescence indicated by a depolarization. The same response to varying external potassium concentrations and valinomycin was also obtained by Sims et al. (87). They further proposed a mechanism in which fluorescence was dependent upon the amount of dye associated with the cell relative to the amount of dye in the surrounding bathing solution. This partitioning mechanism was based on the finding that when a cell was hyperpolarized, the concentration of dye in the bathing medium increased dramatically, while the concentration of the dye remaining associated

with the cell decreased. This cell-associated dye was also found to fluoresce at a much lower intensity and at a different wavelength than the media-associated dye. This relationship led to the suggestion that the dye remaining in the cell formed non-fluorescent complexes. Hladky and Rink (88) provided further evidence for a dye partitioning mechanism, and showed that non-fluorescent complexes occurred within the cell itself and were not simply surface membrane associated phenomena. They also found that in red blood cells the binding of the dye to the cell membrane varied not only with membrane potential, but also with the pH of the cell (89). Work by Freedman and Hoffman (77) further demonstrated this relationship between membrane potential, pH, and dye fluorescence in red blood cells. They were able to form an empirical system which enabled them to calibrate the dye fluorescence response with membrane potential in these cells. From their results they also suggested that another mechanism besides formation of non-fluorescent complexes may be responsible for dye reponse to membrane potential. The equilibrium points for formation of monomer or dimer complexes of the dye were different in red blood cells and in Ehrlich ascites tumor cells. This difference prompted the suggestion that the same mechanism of dye action may not be involved with various cell preparations and dyes, and that each combination of dye with tissue would have to be calibrated individually. Studies of photo-voltage waveforms with numerous different dyes have also shown a wide variation in fluorescence responses depending upon the structure of the dye. A mechanism relating fluorescence to movements of charged dye within the membranes was proposed, but the nature of this movement was unclear and likely different for the various dye structures (84).

Problems Associated with the Dyes

Several problems have been associated with these dyes. DiS-C_3-(5) and some related short chain cyanine dyes have been found to inhibit mitochondrial respiration (93). This same dye also inhibited the calcium dependent potassium channel in red blood cells, but this inhibition was easily blocked by the presence of external potassium (94). In simultaneous microelectrode and dye studies the dye itself was capable of producing slight membrane hyperpolarizations (80). Interference with sodium- potassium membrane fluxes (76, 95) and alteration of other membrane characteristics (95, 96) have also been reported.

Seligmann et al. (85), in working with polymorphonuclear leukocytes, found that use of lower dye concentrations avoided many of the toxic effects which had been associated with the dye when used in higher concentrations. They found that fluorescence increased when polymorphonuclear leukocytes accumulated the dye, and that the mechanism of the fluorescence involved partitioning of the dye, but not dye aggregation or complex formation. Additional studies involving addition of valinomycin to variable sodium, potassium and chloride concentrations led them to state that in polymorphonuclear leukocytes the resting membrane potential is not entirely a function of membrane potassium permeability. They obtained the same results as previous investigators with valinomycin and various external potassium concentrations, but found that depolarizing the cell by raising the external potassium concentration resulted in a decrease in fluorescence. They suggested that the dye was a useful tool for qualitative studies, but warned against equating dye fluorescence changes with membrane potential

changes (85).

Application to Phagocytic Cells

Initial studies using cyanine dyes as probes of membrane characteristics of phagocytic cells have been used with polymorphonuclear leukocytes exposed to various chemotactic agents. Using the dye diO-C_3-(5), Seligmann et al. found that exposure of polymorphonuclear leukocytes to the chemotactic peptide N-formyl methionyl phenylalanine resulted in a diphasic fluorescence response. This reponse was characterized by an initial decrease in fluorescence, followed by an increased fluorescence which stabilized at a new resting level (85, 90). The first change (decreased fluorescence) was enhanced by addition of cytochalasin B (90), but not influenced by alterations in extracellular ion concentrations. No conclusion was made in reference to changes occurring in the membrane potential (85). The second change (increased fluorescence) was found to be inhibited by ouabain, Mg-EGTA, and high external potassium concentrations and was suggested to be caused by a potassium dependent membrane hyperpolarization (85, 90). Similar results were obtained using microelectrodes to study membrane potentials of both polymorphonuclear leukocytes and macrophages exposed to this chemotactic factor (37, 55). Exposure of polymorphonuclear leukocytes to low concentrations of the secretagogues phorbol myristate acetate and ionophore A23187 also resulted in a membrane hyperpolarization as indicated by an increase in dye fluorescence. Higher concentrations of these agents had an inhibitory influence on fluorescence by initially causing a brief hyperpolarization followed by a large depolarization (90). These

results also correspond well with previous microelectrode studies (32).

Using the cyanine dye diS-C_3-(5), Utsumi et al. (91) showed an increase in fluorescence when polymorphonuclear leukocytes were exposed to the lectin concanavalin A, which is known to alter intracellular metabolism. Cholchicine, an antitubulin, and cholesterol ester and cepharanthine, which act to lower the mobility of concanavalin A receptors and fluidity of the membrane, inhibited this response, while increased temperature and cytochalasin B, an inhibitor of microfilament function, acted to enhance it. These results suggested to the investigators that changes in membrane potential may be closely related to both the fluidity of the polymorphonuclear leukocyte membrane and to its cytoskeletal structure. A similar study was done using radiolabeled $[3H]^+$ triphenylmethyl phosphonium ion, a moniter of membrane potential unrelated to the cyanine dyes. Results of exposure of polymorphonuclear leukocytes to concanavalin A and immune complexes resulted in a triphasic response characterized by an initial rapid hyperpolarization, followed by a brief depolarization, then another longer, slow hyperpolarization (67). These results provided further evidence for the usefulness of fluorescent probes as indicators of membrane potential changes in these cells.

Use with Flow Cytometry

Future use of these dyes on phagocytic cells could easily be combined with flow cytometry techniques to allow rapid and precise classification of cells on the basis of membrane potential. A study on peritoneal macrophages stained with the dye acridine orange demonstrated a rapid classification of the cells on the basis of RNA

content by flow cytometry (92). The cyanine dye diO-C_5-(3) was used on lymphocytes to study their reponse to various compounds by flow cytometry. Valinomycin, gramicidin, and concanavalin A all produced effects which were easily detected and interpreted (97). The calibration of dye reponse to membrane potential would enable flow cytometry to be a powerful tool in future studies on the membrane potential characteristics of large numbers of cells.

CHAPTER VI

SCANNING ELECTRON MICROSCOPY OF MACROPHAGES

Scanning electron microscopy has only recently begun to be used as a tool to further characterize the nature of the membranes of phagocytic cells.

Initial Studies - Morphologic Characteristics

The earliest scanning electron microscope observations of normal macrophages were made by Carr et al. (98). They described a surface which usually showed numerous ridge-like structures, with an absence of any notable cellular projections. Hope and Friend (99) saw cells with a wide variety of shapes, irregular surfaces, and finger-like structures coming from the cell. Albrecht et al. (99) described bumpy cell surfaces with low ridges and foot and finger-like processes. Stimulation of these cells by injection of glycerol trioleate or mineral oil into the peritoneal cavity prior to cell collection resulted in cells with a more dramatic appearance. Carr (98) described stimulated cells to be larger than normal with flange and finger-like processes. Warfel and Elberg (100) described cell bodies with numerous membranous structures and large foot-like processes projecting outwards. The presence of ridges, membranes, and foot processes were also confirmed by Albrecht (99). Later observations on thioglycollate and endotoxin stimulated cells showed large, rapidly spreading cells with prominent

membrane ruffling and foot processes. Small cytoplasmic pits, suggested to be pinocytotic vesicles, were also described (102). These and other observations resulted in the description of three characteristic morphological features of phagocytic cell membranes. These are ruffled membranes (lamellopodia), foot-like processes (filopodia or pseudopodia) and finger-like processes (microvilli) (101).

More recently two morphologically distinct alveolar macrophage cell populations have been described in vitro. These consisted of a round cell type and a flatter, more spread out cell type (103). Both types were also found to exist by in situ studies of frozen lung biopsies. The two types may be different shapes assumed by the same cell dependent on the degree of activation or stimulation of the cell (103).

The morphology of cultured macrophages also appears to depend on the surface on which they were cultured. Culturing on glass surfaces resulted in a flat, spread out cell, while culturing on millipore filters yielded a more rounded cell. The comment was made that the millipore filter may provide a surface more like that of the alveolar tissue of the lung, and would provide a more natural view of the cells in vitro which is less distorted than if a glass surface is used (104).

Response to Various Stimuli

Scanning electron microscopy has been used to examine membrane changes in response to certain agents known to effect cell function. Concanavalin A has been shown to greatly decrease the number of surface folds and ruffles in macrophages (105). Cytochalasin B caused a similar decreased ruffling, and caused flat cells to condense into rounded ones

(103, 106). Cholchicine also has caused flat cells to round up. These rounded cells often left behind extensive fine projections which extended to the original cytoplasmic boundaries (103).

Alveolar macrophages have also been exposed to a variety of noxious stimuli and observed with scanning electron microscopy. Cadmium has caused a loss of membrane ruffling (107), while zinc produced condensation of the cytoplasm and subsequent rounding of all the cells (109). Chronic smoke exposure caused an increase in cell surface area with concurrent increases in the number of ruffles and filopodia (108). Studies with quartz and asbestos dust showed that quartz caused loss of ruffling, membrane deterioration, and long slender filopodia (110, 111), while asbestos caused the cells to develop flat, long pseudopodia (111, 112).

Changes During Phagocytosis

Certain aspects of the process of phagocytosis can be viewed by scanning electron microscopy. The major steps in this process involve the attachment of a particle or organism to the cell, engulfment of the particle, formation of a phagocytic vacuole, fusion of the vacuole with a lysosome and subsequent release of lysosomal enzymes into the vacuole to digest the phagocytized particle (113). Scanning electron microscopy has been used to make morphological investigations of the first two steps, attachment and engulfment, on a wide variety of particles and organisms.

Goodall and Thompson (114) did one of the first phagocytosis studies using Acanthamoeba castellanii and latex beads. They found that following attachment of the beads to the membrane surface, a cup

-like struture formed and gradually closed around the bead. The bead was subsequently internalized, leaving an outline of itself under the membrane for a brief period of time. After 25 to 30 minutes, filopodia became shorter and thicker, and much fewer in number. Surface folds and convolutions also became shallower and less extreme. Tizard et al. (115) exposed sensitized red blood cells to peritoneal macrophages and observed that the macrophage membrane slowly spread over and enveloped any attached red blood cells. Parakkal et al. (101) used 2 to 4 micrometer yeast particles and 18 micrometer latex beads and found a fairly characteristic sequence of changes associated with phagocytosis by macrophages. Within 5 minutes they noted numerous filopodia attaching to the particles. These subsequently coalesced to form a ruffled membrane which then engulfed the particle. Phagocytosis was complete by 15 minutes. Phagocytosis of the beads resulted in stretching of the macrophage membrane with gross distortions in its shape due to the interiorized beads. Petty et al. (116) described similar membrane alterations in macrophages exposed to phospholipid vesicles and specific antibody to hapten headgroups of these vesicles. They found that phagocytosis caused both a reduction of surface folds and ruffles and a decrease in total surface area of the macrophage.

Numerous investigators have described the morphological changes in macrophages associated with phagocytosis of bacteria. Walters et al. (117) reported that Bacillus cereus became attached between membrane ridges and was subsequently trapped and engulfed. The macrophage surface soon became smooth and featureless, with an occasional bulge where a phagocytosed bacteria still remained close

to the surface. Staphylococcus aureus became attached by filopodia or lamellopodia, with no smoothing of the cell surface following massive ingestion. Eschericia coli has been found to attach to neutrophils as soon as 5 seconds following exposure, and in many cases were completely phagocytosed within 1 minute. Many cells phagocytosed enough bacteria within only this 1 minute time span that substantial rounding and loss of membrane ruffles occurred (121). Other reports are similar, describing initial attachment and engulfment of bacteria followed by a swelling and loss of membrane ruffles and projections in both macrophages (99, 101, 110, 115, 119, 120), and polymorphonuclear leukocytes (113, 116, 118, 121).

In light of the work which has been done on macrophages with scanning electron microscopy, as well as microelectrode and cyanine dye studies; these techniques would seem to provide the appropriate means by which to study the macrophage plasma membrane under a wide variety of conditions. The purpose of this study will be to determine the characteristics of the normal macrophage membrane and macrophage membranes which have been exposed to P. hemolytica.

CHAPTER VII

MATERIALS AND METHODS

Collection and Preparation of Macrophages

Three 400 to 500 pound, 8 to 10 month old, mixed breed heifers were used as sources of alveolar macrophages. Each animal was restrained in a chute, and its head immobilized with the use of nose tongs and by grasping the tongue. A cannula housing a culture swab was inserted into the trachea. The swab was then used to open the end of the cannula and removed. Polyethylene lavage tubing was passed through the cannula and on into the bronchi of the lung until resistance was felt as it lodged in a small airway. Sixty milliliter boluses of sterile normal saline were placed into the lung via the lavage tubing and immediately withdrawn. A total of 250 ml of saline was used for each lavage. Collected saline containing macrophages was placed on ice in centrifuge tubes. Animals used for collection were alternated, so a given animal was never used more than once every 7 days.

Collected cells were centrifuged at 22° C for 25 minutes at 1000 rpm (275 x $\underline{g}$) in an International Model UV centrifuge with a swinging bucket rotor (IEC, Needham Heights, Massachusetts). The supernatent was discarded and the pellet resuspended in medium consisting of M-199 with 1% L-glutamine and 20% fetal calf serum (Gibco Laboratories, Grand Island, New York). Cells were counted on a hemocytometer and diluted with media to a concentration of $1x10^6$ cells per milliliter.

Preparation of Cells for Microelectrode Impalements

Cultured Macrophages

Two to three milliliters of media containing $1x10^6$ macrophages per milliliter were placed on Leighton tube coverslips and incubated for 30 to 60 minutes at 37° C. Coverslips were washed in Hanks Balanced Salt Solution with calcium (HBSS) (Gibco Laboratories, Grand Island, New York), then placed back into the Leighton tube with 3 to 4 ml of fresh media and capped. Tubes containing coverslips were then incubated at 37° C until ready for use.

Macrophage Suspensions

Three to five milliliters of media containing $1x10^6$ macrophages per milliliter were centrifuged at 22° C for 15 minutes at 1000 rpm (275 x g) in an International Model UV centrifuge. Supernatent was removed, and the pellet transfered to a Beem capsule (Ted Pella Co., Tustin, California), and resuspended in fresh media. Beem capsules were placed into centrifuge tubes and centrifuged for 15 minutes at 1000 rpm. The Beem capsule was then removed and mounted for immediate use for microelectrode impalements.

Preparation of Bacterial Suspensions

P. hemolytica was obtained from lyophilized cultures previously isolated from feedlot cattle. Bacteria were resuspended with 1 ml of Phosphate-buffered saline, streaked onto nutrient agar plates, and incubated in candle jars at 37° C for 24 hours. Agar consisted of BHI

(Difco Laboratories, Detroit, Michigan) supplemented with 5% citrated blood, 1% sterile filtered yeast, and 1% horse serum. Pure colonies were removed from the plates and suspended in HBSS. Bacterial concentration was determined by spectrophotometer (Spectrometer 20, Bausch and Lomb Inc., Rochester, New York) O.D. readings and comparison with a standard curve. Solutions were diluted to give a final concentration of $1x10^7$ bacteria per milliliter.

Staphylococcus aureus was obtained from a fresh culture isolated from a clinical case. Colonies were suspended in HBSS and diluted to an approximate concentration of $1x10^7$ bacteria per milliliter.

Preparation of Formalized Pasteurella hemolytica

Fresh colonies prepared as above were suspended in 10% formalin for 10 days. Bacteria were centrifuged in a Sorvall RC2-B centrifuge with an HB-4 rotor (DuPont, Newton, Connecticut) at 2° C for 20 minutes at 12,500 rpm (25,000 x g). Formalin was removed and discarded, and the bacterial pellet was resuspended in HBSS. Bacteria were washed with HBSS in this manner 4 times. The final suspension of bacteria in HBSS was stored at 4° C until ready for use.

Macrophage Preparation for Scanning Electron Microscopy

Five different treatments were made and prepared for observation with scanning electron microscopy. Treatments consisted of normal macrophages, macrophages exposed to P. hemolytica in both 10:1 and 20:1 bacteria to macrophage ratios, macrophages exposed to formalized P. hemolytica in a 20:1 bacteria to macrophage ratio, and macrophages

exposed to Staphylococcus aureus in a 20:1 bacteria to macrophage ratio. Approximately 2.5 x 10^5 macrophages in M-199 with 20% fetal calf serum and HBSS were used for each treatment group in a total volume of 3 to 4 ml. Each treatment was incubated at 37° C for various intervals of time. The normal cells were incubated for 60 minutes. The macrophages exposed to 20 P. hemolytica per macrophage were incubated for between 10 to 60 minutes. Samples were made at each 10 minute interval within this range. All other groups were sampled after both 30 minutes and 50 minutes of incubation. Following incubation, the mixtures were spun for 20 to 30 seconds in a Model 5414 Eppendorf Microcentrifuge (Brinkman Instruments Inc., Westbury, New York), the supernatent was removed, and the cells were fixed in 2% cacodylate buffered glutaraldehyde for 90 minutes. This was followed by three 20 minute washes in sodium cacodylate. Cells were then placed on coverslips which had been coated with 1% polylysine and allowed to sit for 20 minutes. 2% glutaraldehyde was then added for 15 minutes, followed by three 20 minute washes in distilled water. Cells mounted on coverslips were dehydrated with sequential 20 minute washes in 50%, 70%, 90%, 95%, and three 100% solutions of ethanol. Cells were then critical point dried with carbon dioxide in a Samdri pvt 3 critical point dryer (Tousimis Research Corp., Rockville, Maryland), and outgassed under high vacuum in a Denton DV-502 Vacuum Evaporator (Denton Vacuum Co., Cherry Hill, New Jersey). Coverslips were mounted on aluminum stubs and coated with a 200 Angstrom thick layer of gold-palladium with a Hummer II sputter coater (Technics, Alexandria, Virginia) just prior to viewing.

Experimental Procedures

Microelectrode Impalements - Cultured Macrophages

Macrophages attached to Leighton tube coverslips were used for impalements within 6 hours of collection. Coverslips were attached to an inverted micromanipulator and suspended in a petri dish containing HBSS which was mounted on a microscope stage (Nikon Inc., Garden City, New York). Microelectodes were pulled from GCF-120-6 microfilament glass (A-M Systems Inc., Everett, Washington) on a micropipette puller (Industrial Science Associates Inc., Ridgewood, New York). Microelectrodes used for impalements had tip diameters of approximately .4 to .8 micrometers and approximate tip resistances of 10^{15} to 10^{20} ohms. These were back-filled with 250 mM potassium chloride, mounted in a micromanipulator which was attached to a hydraulic drive mechanism, and connected to a 3431J Electrometer amplifier (Burr Brown, Tucson, Arizona) by a silver-silver chloride wire which was grounded through an agar bridge connecting the HBSS bathing solution to a calomel cell. Membrane potential readings were made on a digital panel meter and a chart recorder (Linear Instrument Corp., Irvine, California). Recordings were made on only normal, attached macrophages. Impalement criteria which were required before a recording would be accepted for this study were a rapid potential change upon cell impalement, a stable new potential for at least two minutes, and a rapid return to within 3 to 4 mV of baseline values upon removal of the microelectrode from the cell.

Microelectrode Impalements

- Suspended Macrophages

Beem capsules containing a pellet of macrophages were prepared as previously described. These were mounted below a micromanipulator containing a microelectrode which was arranged as before. Impalements were made by advancing the microelectrode into the cell pellet. Recordings were made on normal cells and cells which had been incubated with a 20:1 concentration of P. hemolytica at 37° C for 60 minutes.

Cyanine Dye Measurements

Four different treatments, each containing 3 samples, were run on seven separate cell collections. Each sample consisted of 2.5 x 10^5 macrophages in M-199 with 20% fetal calf serum and HBSS. Treatment 1 consisted only of macrophages. P. hemolytica were added to treatment 2 in a 20:1 bacteria to macrophage ratio, and to treatment 3 in a 10:1 bacteria to macrophage ratio. Treatment 4 contained formalized P. hemolytica in a 20:1 bacteria to macrophage ratio. The volume for all treatment mixtures was 1 ml, with an additional 60 microliters of the cyanine dye diO-C_5-(3) (Molecular Probes, Plano, Texas) added to each. Mixtures were prepared in microcuvettes with the cell suspensions added just prior to the first reading. Suspensions were stirred prior to each reading and kept in subdued light at 26° C between readings. Readings were made at 5 minute intervals from 0 to 45 minutes. Percent transmittance values were recorded on an Aminco-Bowman Spectrophotofluorometer (American Instrument Co., Silver Spring, Maryland) with the excitation wavelength set at 460 nm, and the emission wavelength at 510 nm. Multiplier settings of .1 or .03

were set at the beginning of each run, and remained at that setting for each individual group of experiments.

Scanning Electron Microscope Observations

Observations of the specimens prepared for scanning electron microscopy were made with a JSM 35-U Scanning Electron Microscope (Jeol Ltd., Tokyo, Japan). Photographs were taken at 23 kV with Polaroid Type 55 Land film (Polaroid Corp., Cambridge, Massachusetts).

Data Analysis

Statistical analysis consisted of Analysis of Variance for all treatment groups in the dye experiments, with unpaired t-tests of those treatments in time intervals showing significant differences with the analysis of variance. Each treatment in a single time interval was compared by putting values on a percent scale of the peak fluorescence for that treatment over all time intervals.

Comparisons of microelectrode groups and treatments were done with unpaired t-tests.

CHAPTER VIII

RESULTS

Microelectrodes

Two different microelectrode techniques were used on normal macrophages. Cells which were attached to Leighton tube coverslips and impaled showed two different membrane potential results. Four cells had membrane potentials ranging from -11 mV to -13 mV with an average value of -11.9 mV. Two cells, however, had positive membrane potentials with an average of +29 mV. Two of these cultured cells spontaneously hyperpolarized upon impalement. Normal cells which were pelleted and randomly impaled yielded three distinct types of responses. Five cells had an average membrane potential of -11.7 mV with a range of -10.5 mV to -13 mV. Two other cells had membrane potentials of -22 mV and -26 mV. A single cell showed a large negative membrane potential of -56 mV. One cell in this group spontaneously hyperpolarized upon impalement. The average for all but the -56 mV cell was -15.2 mV. A two tailed t-test between the mean of the pelleted groups and the mean for the cells impaled while attached to coverslips showed no significant difference between the means of these two groups ($p > .05$).

Pelleted suspensions of macrophages which had been exposed to a 20:1 suspension of P. hemolytica for 60 minutes were randomly impaled. The membrane potential of 11 impaled cells ranged from -13 mV to -38 mV with an average membrane potential of -25.1 mV. A two

tailed t-test showed a significant difference ($p < .005$) between the mean of the normal cells (-15.2 mV), and the mean of the infected cells (-25.1 mV).

Cyanine Dye

In the normal group as well as the 10:1 and 20:1 P. hemolytica to macrophages treatments, and the 20:1 formalized P. hemolytica to macrophage group, flourescence began at initially high values and progressively decreased (Figure 1). The only increase noted was a slight one in the formalized group between the 40 and 45 minute time intervals. The fluorescence of the normal group decreased at a greater rate than the fluorescence in the three treatment groups. Comparing fluorescence intensity between the groups, the formalized group showed the greatest fluorescence for all groups from 0 to 20 minutes, while the 20:1 P. hemolytica: macrophage group showed the greatest fluorescence from 20 to 45 minutes. The 10:1 P. hemolytica: macrophage treatment group remained consistently lower than both of the 20:1 treatments, but still fluoresced at a substantially greater level than the normal group.

Analysis of variance of the treatments at each time interval showed no significant differences between the groups from 0 to 15 minutes ($p > .05$). A significant difference between treatments was indicated for each time interval from 20 minutes to 45 minutes ($p < .05$). All the treatments at each of these time intervals were compared to one another with two tailed t-tests. The 20:1 P. hemolytica: macrophage group showed a significant difference between the normal group at 25 minutes ($p < .05$) and showed increasing significance with

Figure 1 Membrane Fluorescence Intensity with Time, Normal vs. All Treatments. Fluorescence of normal macrophages (o); macrophages incubated with a 20:1 ratio of P. hemolytica for 0 - 45 minutes (•); macrophages incubated with a 10:1 ratio of P. hemolytica for 0 - 45 minutes (□); and macrophages incubated with a 20:1 ratio of formalized P. hemolytica for 0 - 45 minutes (■).

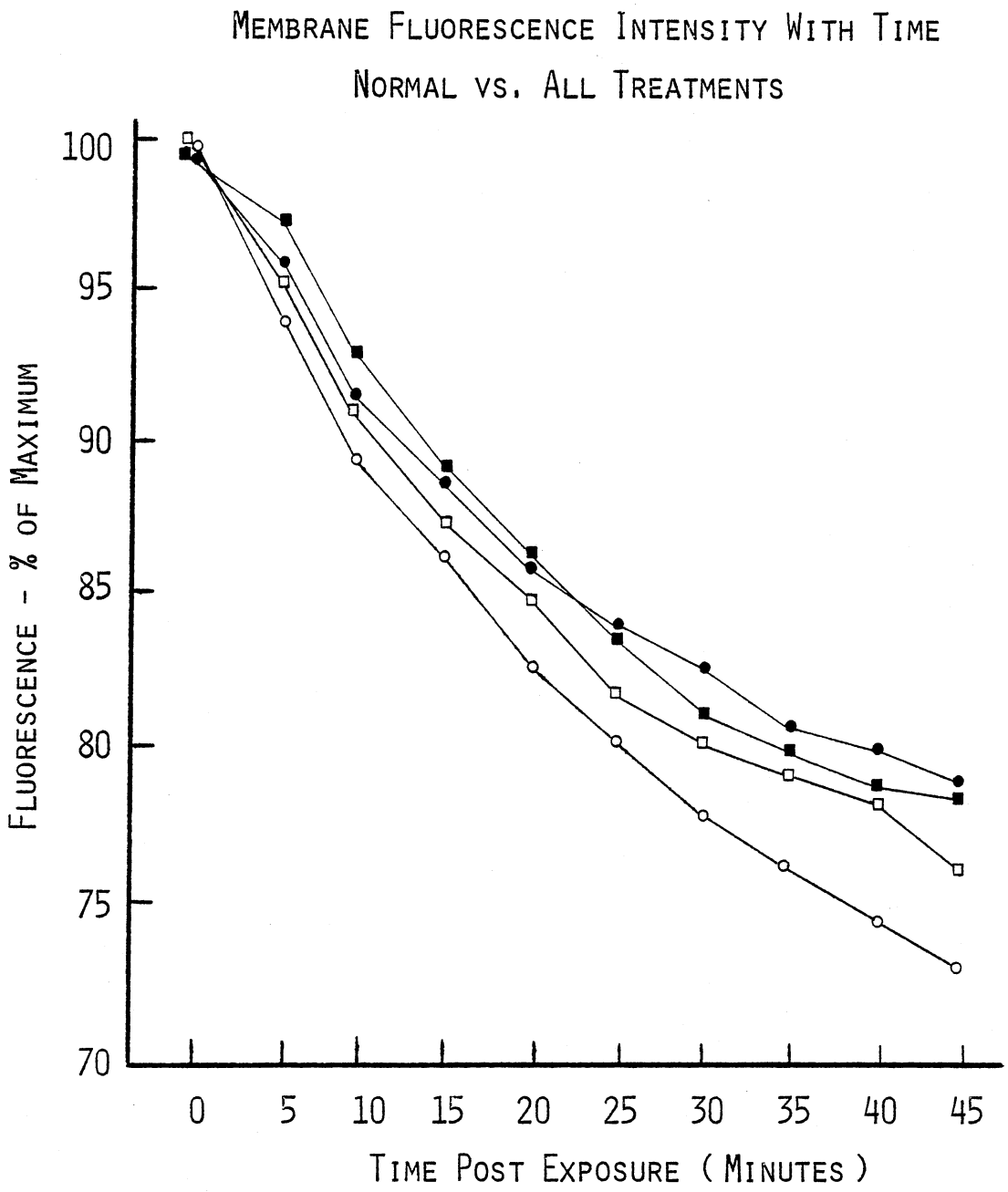

MEMBRANE FLUORESCENCE INTENSITY WITH TIME
NORMAL VS. ALL TREATMENTS
FLUORESCENCE - % OF MAXIMUM
100
95
90
85
80
75
70
0
5
10
15
20
25
30
35
40
45
TIME POST EXPOSURE (MINUTES)

Figure 2 Membrane Fluorescence Intensity with Time, Normal vs. 20:1 Pasteurella hemolytica. Fluorescence of normal macrophages (o), and macrophages incubated with a 20:1 ratio of P. hemolytica for 0 - 45 minutes (•). Standard errors and time intervals showing a significant difference between groups ($p < .05$), are indicated by vertical bars.

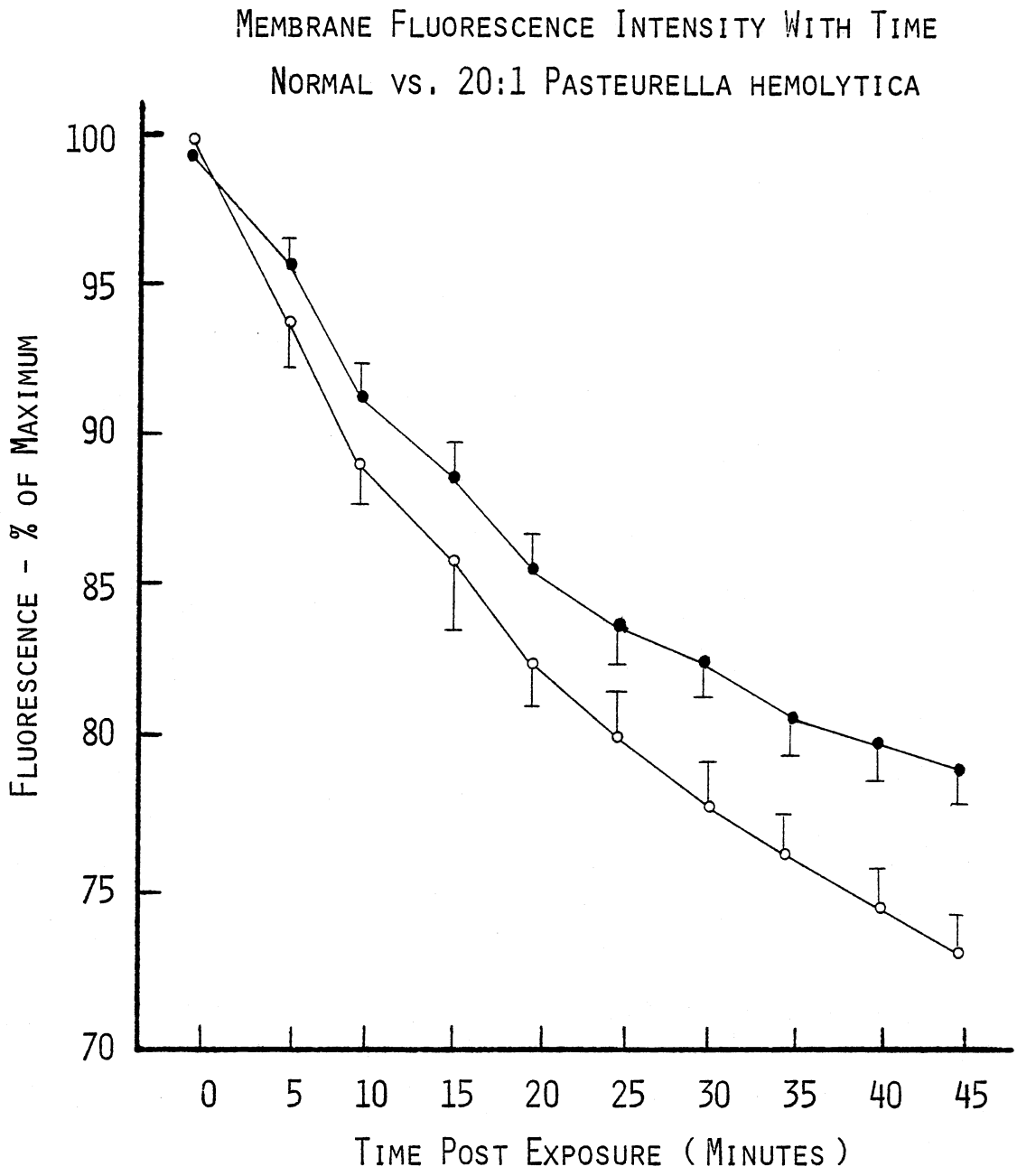

MEMBRANE FLUORESCENCE INTENSITY WITH TIME
NORMAL VS. 20:1 PASTEURELLA HEMOLYTICA
FLUORESCENCE - % OF MAXIMUM
100
95
90
85
80
75
70
0
5
10
15
20
25
30
35
40
45
TIME POST EXPOSURE (MINUTES)

Figure 3 Membrane Fluorescence Intensity with Time, Normal vs. 10:1 Pasteurella hemolytica. Fluorescence of normal macrophages (o), and macrophages incubated with a 10:1 ratio of P. hemolytica for 0 - 45 minutes (□). Standard errors and time intervals showing a significant difference between groups ($p < .05$), are indicated by vertical bars.

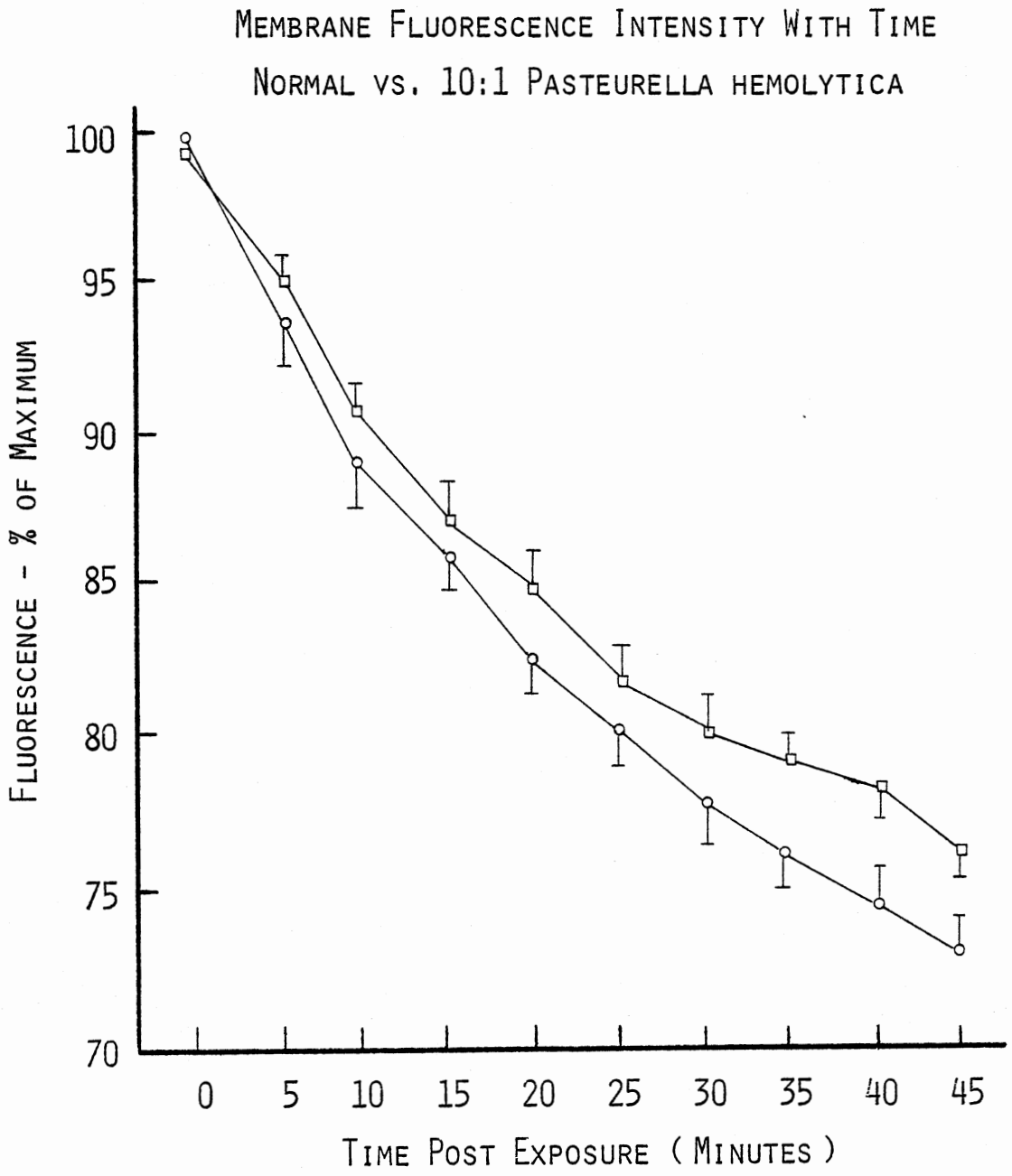
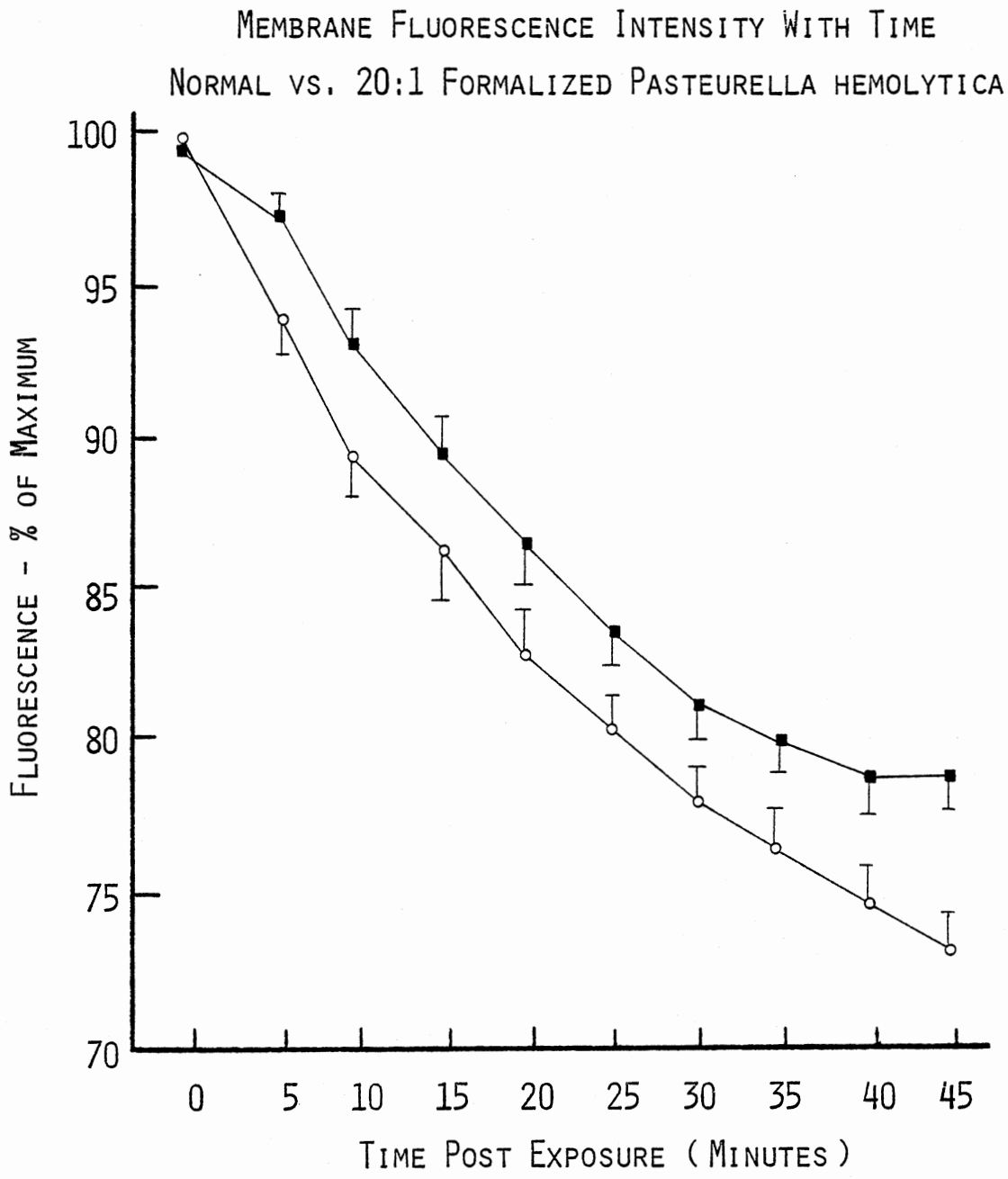

MEMBRANE FLUORESCENCE INTENSITY WITH TIME
NORMAL VS. 10:1 PASTEURELLA HEMOLYTICA
FLUORESCENCE - % OF MAXIMUM
100
95
90
85
80
75
70
0
5
10
15
20
25
30
35
40
45
TIME POST EXPOSURE (MINUTES)

Figure 4 Membrane Fluorescence Intensity with Time, Normal vs. 20:1 Formalized Pasteurella hemolytica. Fluorescence of normal macrophages (o), and macrophages incubated with a 20:1 ratio of formalized P. hemolytica for 0 - 45 minutes (■). Standard errors and time intervals showing a significant difference between groups ($p < .05$), are indicated by vertical bars.


MEMBRANE FLUORESCENCE INTENSITY WITH TIME
NORMAL VS. 20:1 FORMALIZED PASTEURELLA HEMOLYTICA
FLUORESCENCE - % OF MAXIMUM
100
95
90
85
80
75
70
0
5
10
15
20
25
30
35
40
45
TIME POST EXPOSURE (MINUTES)

time ($p < .001$ at 45 minutes) (Figure 2). The 10:1 *P. hemolytica*: macrophage group showed a significant increase over the normal group at only the 40 minute ($p < .05$) and 45 minute ($p < .001$) time intervals (Figure 3). The 20:1 formalized *P. hemolytica*: macrophage treatment group showed a significantly increased fluorescence over the normal group at each time interval from 20 minutes ($p < .05$) to 45 minutes ($p < .001$) (Figure 4). No significant difference between each of the three treatment groups was detected at any time interval.

Scanning Electron Microscopy

Observations of the alveolar macrophage with the scanning electron microscope showed that the surfaces of these cells appeared similar to the surfaces described previously for other normal macrophages (98-101). The normal alveolar macrophages were fairly heterogeneous in morphology, but several characteristic features dominated their surfaces (Figure 5). Long, thin lamellopodia with sharp edges were very distinct, as were other long surface folds. Microvilli were present, but not nearly as extensive as the lamellopodia and other surface folds.

Exposure of macrophages to *P. hemolytica* resulted in a distinct sequence of changes in the surface morphology of the macrophages. These changes appeared to depend primarily upon the length of exposure to *P. hemolytica*. The general sequence of morphological changes in the membrane appeared in macrophages exposed to a 20:1 ratio of *P. hemolytica*: macrophages over a 60 minute period of time. First there was a reduction in the number of lamellopodia, with a concurrent increase in microvilli (Figures 6-7). The remaining lamellopodia tended to be flatter and more rounded than before. Surface projections then

began to become more globular in nature, while the surface of the cells smoothed out (Figures 8-10). These globular projections eventually flattened out to give the cell a rough irregular surface which contained few prominent projecting structures (Figures 11-13). Cells then tended to develop a smoother surface, with the occurrence of occasional long, thin microvilli (Figures 14-15). Finally the cell became round with a very smooth surface which was interrupted only by frequent pitted areas and a few, short, thick remnant microvilli (Figure 16).

TABLE I

MAJOR MORPHOLOGIC FEATURES OF MACROPHAGES EXPOSED TO A 20:1 RATIO OF PASTEURELLA HEMOLYTICA

Time Interval	Cell Surface	Lamellopodia	Microvilli
0 minutes	numerous projections	+++ thin and fine	+ thin and fine
10 minutes	numerous projections	++ short and wide	++ thick and short
20 minutes	numerous projections	+ short and globular	+++ thick and short
30 minutes	globular projections	+ short and flat	+ short and blunt
40 minutes	rough irregular	+ flat and round	not apparent
50 minutes	hilly smooth	not apparent	+ thin and sharp
60 minutes	smooth pits present	not apparent	not apparent

Quantitative estimates of numbers present as compared to the average normal macrophage are greater than 70% (+++), 30 to 70% (++), and less than 30% (+).

At most time intervals in the 20:1 P. hemolytica: macrophage treatment examples of several different stages in this sequence of morphological changes could be seen. By 60 minutes, however, the smooth pitted cell type was found to be predominate. A few giant cells were also noted in all the time interval samples following 30 minutes of exposure.

The 20:1 formalized P. hemolytica: macrophage cells showed changes with time which were similar to those seen with exposure to live P. hemolytica (Figures 17-18), however, there was one notable exception. This exception was the absence of any round pitted cells in any of the sampled time intervals (30 and 50 minutes). Features of the predominate cell type present at each sampled time interval are given below.

TABLE II

MAJOR MORPHOLOGIC FEATURES OF MACROPHAGES EXPOSED TO A 20:1 RATIO OF FORMALIZED PASTEURELLA HEMOLYTICA

Time Interval	Cell Surface	Lamellopodia	Microvilli
30 minutes	numerous projections	++ short and round	+ thick and short
50 minutes	smooth irregular	not apparent	not apparent

Quantitative estimates of numbers present compared to the average normal macrophage are greater than 70% (+++), 30 to 70% (++), and less than 30% (+).

Macrophages exposed to a 10:1 P. hemolytica: macrophage ratio of bacteria showed changes which were similar to the 20:1 ratio groups (Figures 19-20). The round, pitted cell type was observed, but it was much less frequent than in the groups exposed to the 20 live P. hemolytica per macrophage. Fewer than 20% of the cells were estimated to show this morphologic characteristic at 50 minutes post incubation. Major morphological features at the 30 and 50 minute time intervals are seen below.

TABLE III

MAJOR MORPHOLOGIC FEATURES OF MACROPHAGES EXPOSED TO A 10:1 RATIO OF PASTEURELLA HEMOLYTICA

Time Interval	Cell Surface	Lamellopodia	Microvilli
30 minutes	numerous projections	+++ long and thin	++ thin
50 minutes	rough irregular	++ short and thick	+ thin and fine

Quantitative estimates of numbers present as compared to the average normal macrophage are greater than 70% (+++), 30 to 70% (++), and less than 30% (+).

Macrophages exposed to 20 Staphylococcus aureus per cell were found to undergo minimal changes with time (Figures 21-22). No obvious morphological differences were noted in cells which had been exposed for 30 minutes compared to those exposed for 50 minutes (see Table IV).

TABLE IV

MAJOR MORPHOLOGIC FEATURES OF MACROPHAGES EXPOSED TO A 20:1 RATIO OF STAPHYLOCOCCUS AUREUS

Time Interval	Cell Surface	Lamellopodia	Microvilli
30 minutes	numerous projections	+++ long and fine	++ long and thin
50 minutes	numerous projections	++ long and fine	+ long and thin

Quantitative estimates of numbers present as compared to the average normal macrophage are greater than 70% (+++), 30 to 70% (++), and less than 30% (+).

Figure 5 Normal macrophages - Lamellopodia are long, thin, and prominent (large arrow). Microvilli are sharp and distinct (small arrow). 7800x.

Figure 6 20:1 Pasteurella hemolytica: macrophages, 10 minutes - Microvilli are the most prominent feature. These are short and thick. Lamellopodia are not as distinct as previously. 7800x.

Figure 7 20:1 Pasteurella hemolytica: macrophages, 20 minutes - Microvilli are still very distinct. Some are beginning to coalesce to form larger, thicker projections. 7200x.

Figure 8 20:1 Pasteurella hemolytica: macrophages, 20 minutes - Microvilli and lamellopodia have become thick and globular. Edges on all projecting structures have become rounded. 7200x.

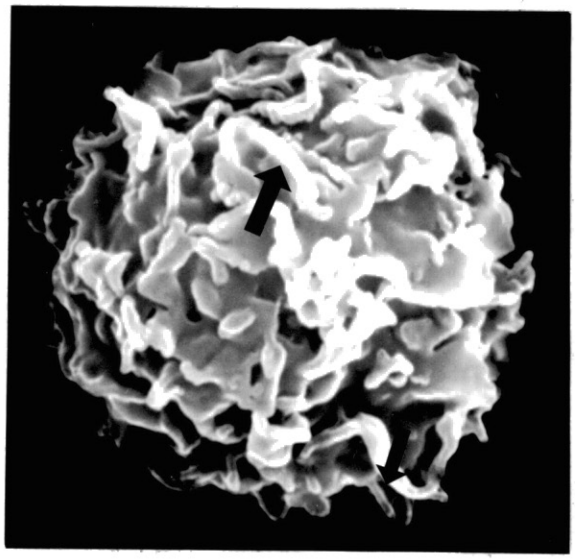


Figure 5

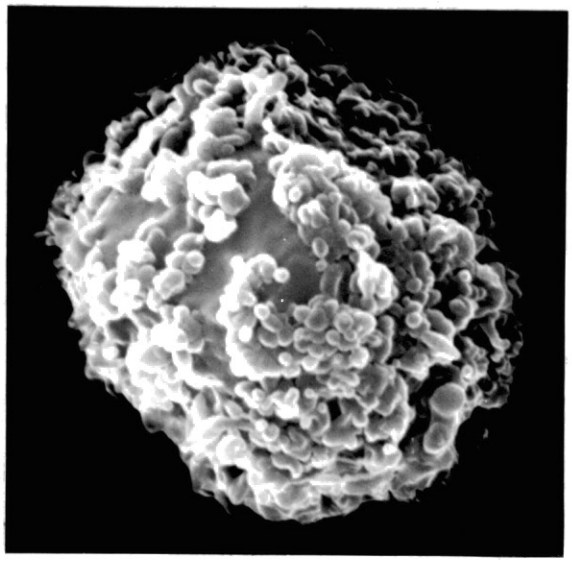


Figure 6

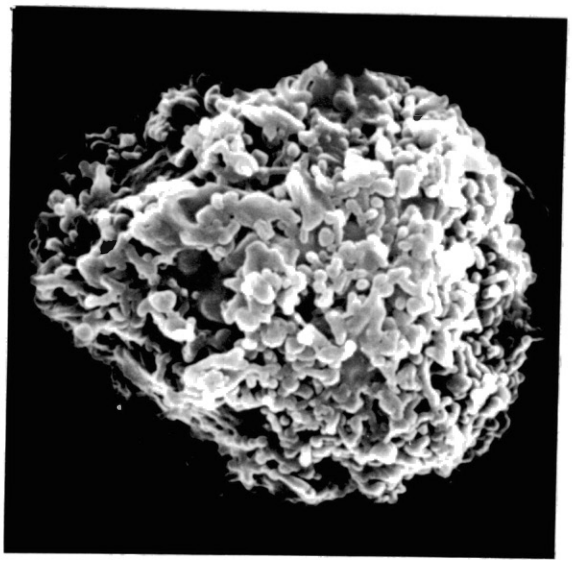


Figure 7

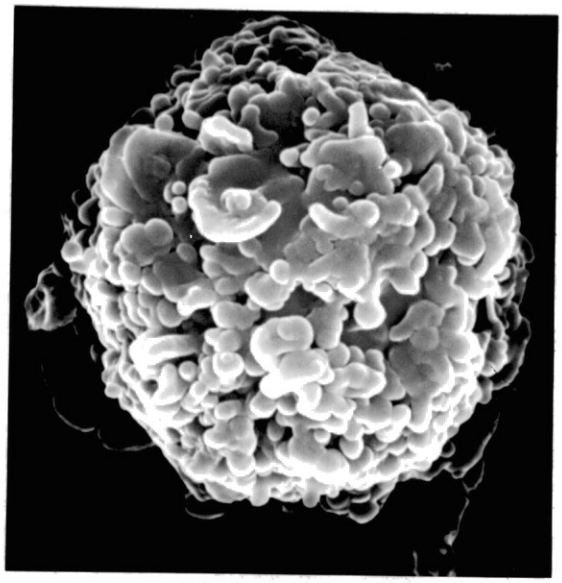


Figure 8

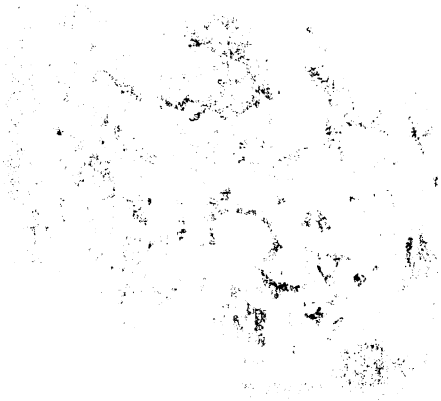


Figure 9 20:1 Pasteurella hemolytica: macrophages, 20 minutes - Some fine microvilli are still present, others have formed into more rounded structures. Lamellopodia are thick, short, and flattened. 7200x.

Figure 10 20:1 Pasteurella hemolytica: macrophages, 30 minutes - The entire surface is composed of globular condensations of plasma membrane. No sharp, distinct microvilli or lamellopodia are seen. 7800x.

Figure 11 20:1 Pasteurella hemolytica: macrophages, 40 minutes - The surface is very irregular, and becoming smooth. Some short, fine microvilli are occasionally projecting from the surface. 7200x.

Figure 12 20:1 Pasteurella hemolytica: macrophages, 40 minutes - The surface is becoming progressively smoother. A small number of sharp, distinct microvilli are still present. 7200x.

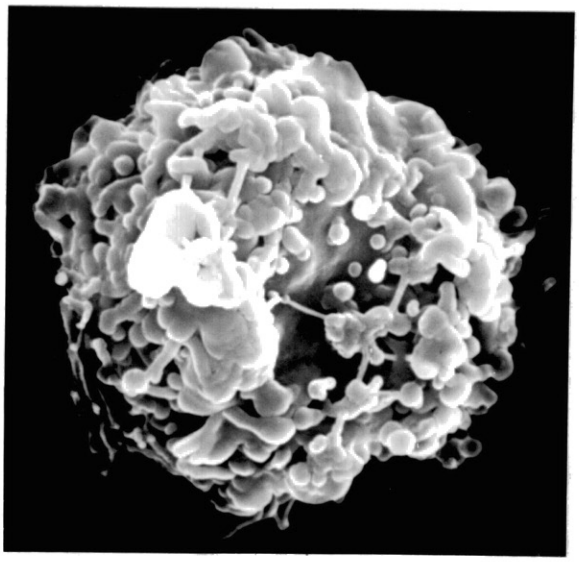


Figure 9

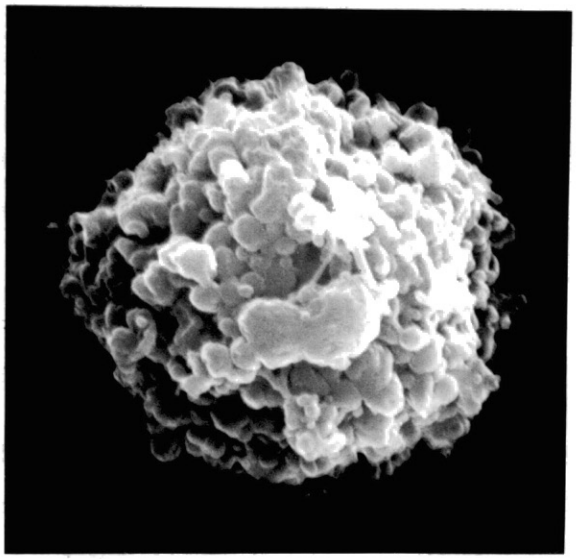


Figure 10

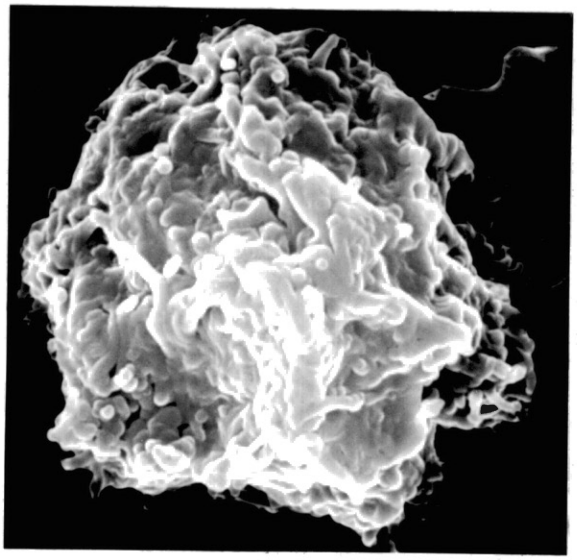


Figure 11

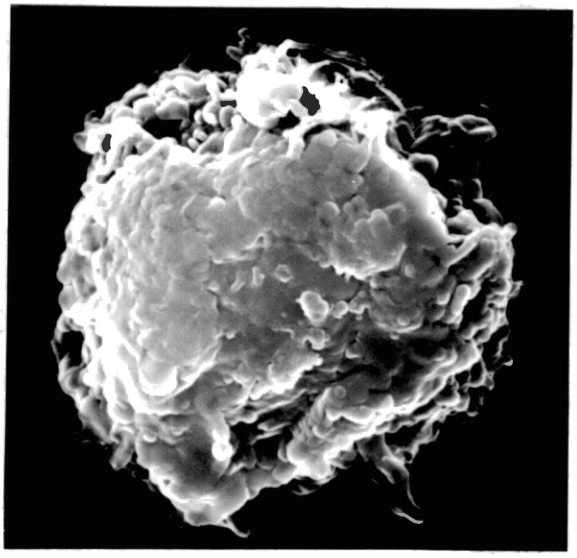


Figure 12

Figure 13 20:1 Pasteurella hemolytica: macrophages, 60 minutes - Microvilli are not noticeable. Membrane projections are not present to any significant degree. The surface is composed of short, rounded, raised areas. 7800x.

Figure 14 20:1 Pasteurella hemolytica: macrophages, 50 minutes - Microvilli are still prominent, the surface is becoming progressively more regular and smooth. 7800x.

Figure 15 20:1 Pasteurella hemolytica: macrophages, 40 minutes - Microvilli are the only prominent surface projections. These are thin and short. The surface of the macrophage is smooth, rounded, and irregular. 7800x.

Figure 16 20:1 Pasteurella hemolytica: macrophages, 50 minutes - The surface is smooth. Occasional short microvilli are present, interspersed with small membrane pits. 7200x.

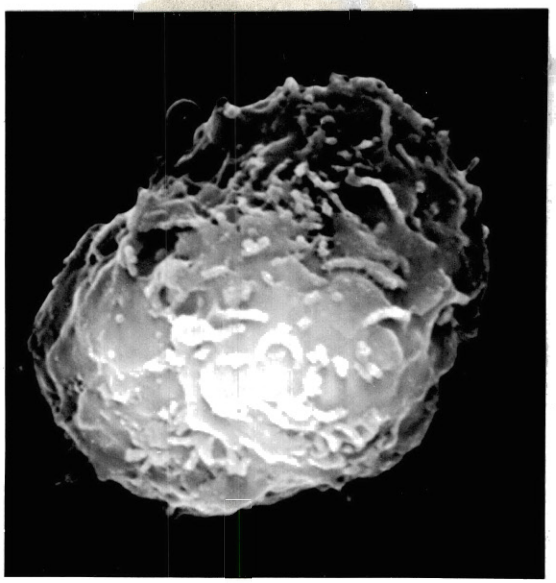


Figure 13

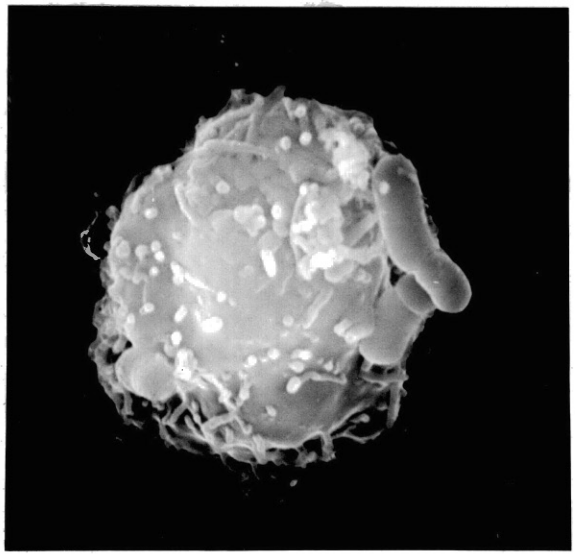


Figure 14

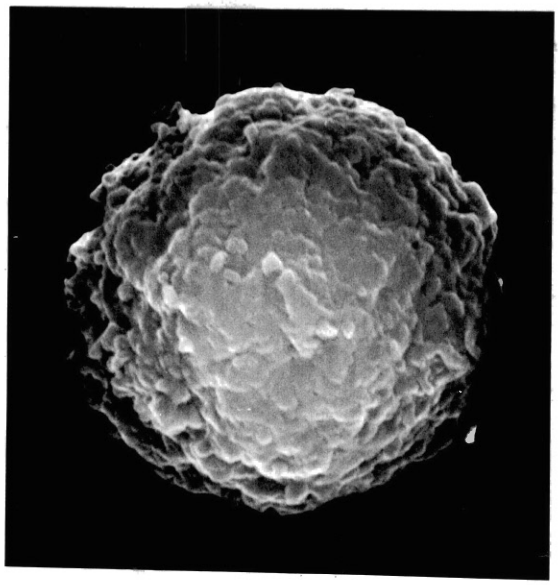


Figure 15

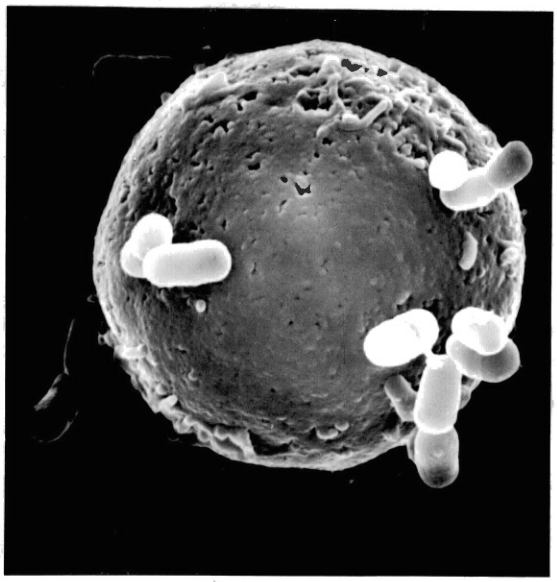


Figure 16

Figure 17 20:1 formalized Pasteurella hemolytica: macrophages, 30 minutes - Microvilli and lamellopodia are distinct and prominent. Some condensation and rounding of these surface projections has occurred. 7800x.

Figure 18 20:1 formalized Pasteurella hemolytica: macrophages, 50 minutes - Microvilli and lamellopodia are absent. The surface is smooth and irregular. Flattened ridges separated by depressed areas are the principle morpholologic surface features. 7800x.

Figure 19 10:1 Pasteurella hemolytica: macrophages, 30 minutes - The predominant feature is numerous, thickened microvilli. Lamellopodia are short and rounded. 7800x.

Figure 20 10:1 Pasteurella hemolytica: macrophages, 50 minutes - Membrane projections are not numerous, these consist predominately of microvilli. The surface is irregular, rough, and rounded. 7800x.

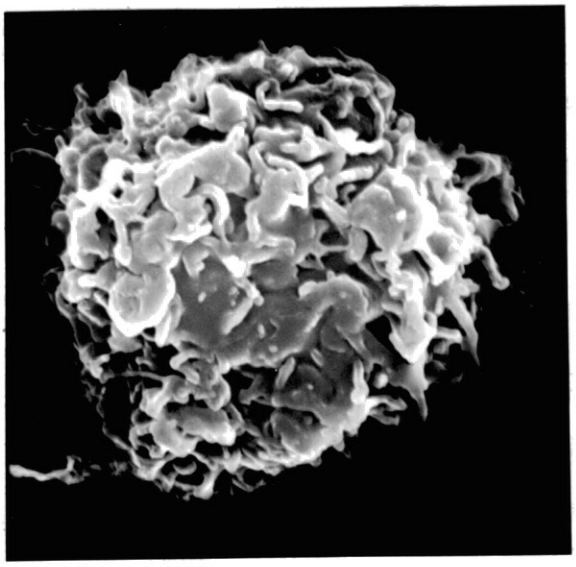


Figure 17

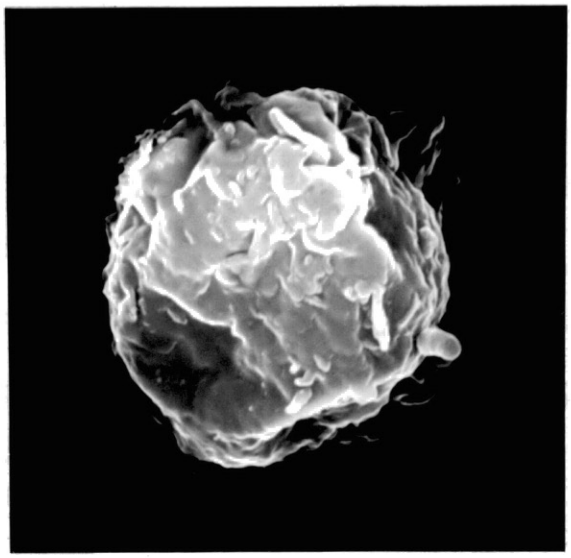


Figure 18

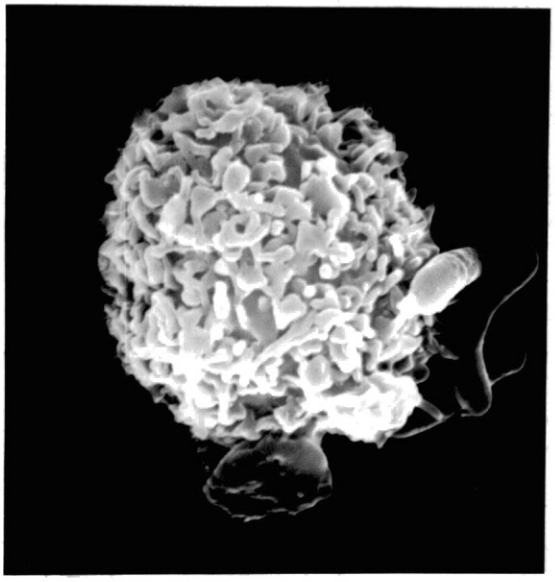


Figure 19

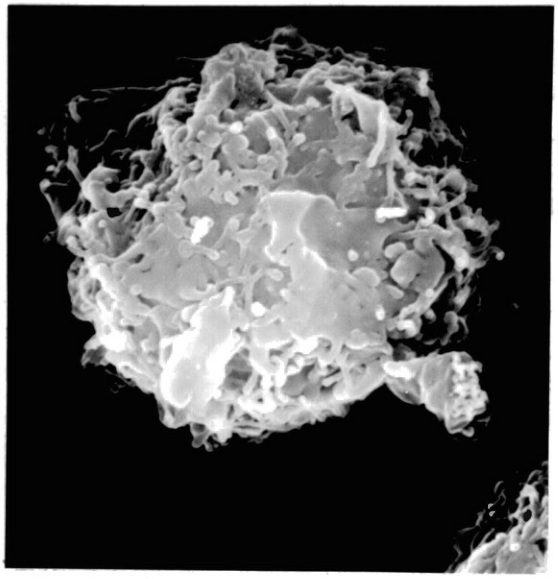


Figure 20

Figure 21 20:1 Staphylococcus aureus: macrophages, 30 minutes - Lamellopodia are long, tall and distinct. Few microvilli are present. 7800x.

Figure 22 20:1 Staphylococcus aureus: macrophages, 50 minutes - Lamellopodia are still prominent, though not as numerous as previously. These are long and thin. The underlying surface is smooth and irregular. 7800x.

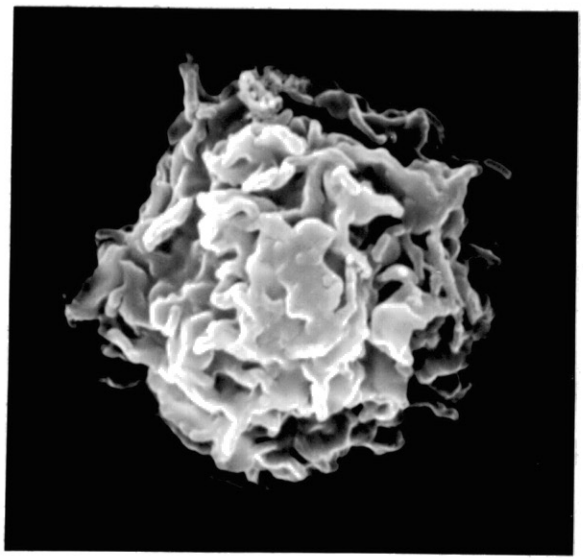


Figure 21

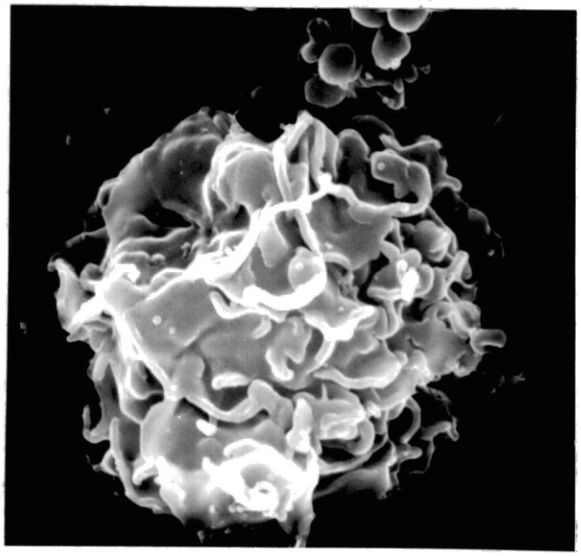


Figure 22

CHAPTER IX

DISCUSSION

In the present study an investigation was made into the nature of changes in the macrophage plasma membrane which might be caused by a cytotoxic factor which has been reported to be associated with P. hemolytica (22, 23, 24). Three approaches were taken: 1) Monitering membrane potential with microelectrodes, 2) Monitering membrane potential with a membrane potential sensitive fluorescent dye, and 3) Investigating morphologic changes in the macrophage membrane with scanning electron microscopy.

Microelectrodes

Results of microelectrode impalements showed a significant difference in membrane potential between normal macrophages and those exposed to P. hemolytica. Two different microelectrode techniques were used in this study for two principle reasons. Impaling cultured macrophages proved to be a time consuming and tedious process. The nature of the membrane, the brief period of time allowed for attachment, and the desire to make recordings within 6 hours of collection were all factors in complicating the impalement of these cultured cells. Other investigators have also encountered problems with impaling these cells (36, 67) and have dealt with the issue in a variety of ways. Most other microelectrode studies of macrophages have involved

impalement of blood mononuclear cells (37), peritoneal macrophages (32), or foreign body induced cells (36), all of which were normally cultured for at least several days before impalements were made. Although this longer culturing period allows for firm attachment and an increase in the size of the cell, chances are increased that the cell may loose some of the characteristics it possessed in situ.

Reports have also indicated that exposure to P. hemolytica causes detachment of macrophages from the surface on which they are cultured (8, 23). Microelectrode impalements on detached or loosely adhered cells would be extremely difficult. This problem could have possibly been avoided by using a resin on the culturing surface (32), but the presence of resin with the cells adds another modifying factor to a system which is already quite alien to the normal environment of the macrophage.

The technique by which macrophages were loosely pelleted and impaled by simply advancing a microelectrode into the pellet was devised to avoid the previously mentioned problems. A major concern was whether the process of pelleting the cells would have an effect on the membrane potential of individual cells. The -11.9 mV average for cells impaled on coverslips was very close to the -11.7 mV average for pelleted cells. A two tailed t-test showed no significant difference between them. The values obtained by both techniques also compared favorably with the findings of other investigators (32-38, 40).

Though only 14 recordings of normal cells were used for this study, three distinct types of values were obtained. The majority of the cells had membrane potentials between -10 mV to -13 mV. Two cells were found to have positive membrane potentials (+26 mV), and one cell had a large

negative potential (-56 mV). Gallin et al. have reported the presence of two distinct peritoneal macrophage cell populations on the basis of membrane potential and their current-voltage relationships (38). The values for their two groups correspond fairly well to the low negative and high negative potential cells measured in this study. They make no mention of any positive potential cells. Kouri et al., however, have reported encountering these cells, but declined to comment on what significance they might have (34). This present study can add no information as to the nature of these cells, but only confirms that some macrophages do show positive membrane potentials. Spontaneous hyperpolarizations similar to those previously described by Gallin (32) were occasionally encountered. All of these appeared to be associated with penetration of the membrane by the microelectrode.

In contrast to the normal macrophages, only the pellet technique was used for impaling the macrophages which had been exposed to P. hemolytica. The range of values obtained was considerably greater than that for the majority of the normal cells. Membrane potentials varied from -13 mV to -38 mV, with an average of -25.1 mV. These values were significantly greater than the -15.2 mV average for the normal cells, indicating that a hyperpolarization of the cells occurred due to exposure to P. hemolytica. Responses similar to these have been obtained by Kouri et al. (34) when macrophages were exposed to phagocytosible particles. This suggests that the changes noticed in this study could have been due to membrane changes associated with phagocytosis. The other most likely explanation for these hyperpolarizations would be that they were caused by some substance associated with P. hemolytica which interacted with the macrophages

(such as a cytotoxin). Cyanine dye studies were undertaken to more accurately characterize this response.

Cyanine Dye

Results obtained with the fluorescent dye diO-C_5-(3) support the idea that the hyperpolarizations noticed with microelectrodes in the 20:1 P. hemolytica exposed cells were due to membrane changes associated with phagocytosis. All three treatment groups were found to fluoresce at a greater intensity than normal cells at every time interval in this study. Statistically significant increases in the intensity of fluorescence over the normal untreated macrophages were found for the 20:1 formalized P. hemolytica: macrophage treatment group by 20 minutes, 20:1 P. hemolytica: macrophage group at 25 minutes, and the 10:1 P. hemolytica: macrophage group at 40 minutes. It would appear that exposure of macrophages to phagocytosible particles causes an increase in fluorescence, indicating a hyperpolarization of the cells.

The process of phagocytosis begins within seconds of exposure of neutrophils to E. coli (121). With 100:1 ratios of microorganisms to macrophages and neutrophils this process is reported to reach a maximum at 45 minutes (118). Though not reaching significant levels until between 20 and 40 minutes, fluorescence in this study was noted to increase over normal untreated cell fluorescence for all treatment groups by less than 5 minutes following exposure to phagocytosible particles. These findings indicate a clear trend of an increasing fluorescence during active phagocytosis which is compatible with the course of events in relation to time found in previous studies on

phagocytosis.

Though not shown to be statistically significant, both of the 20:1 P. hemolytica: macrophage ratio groups had consistently greater fluorescence than the 10:1 P. hemolytica: macrophage group at all time intervals. Several possible causes for this increased fluorescence with higher numbers of bacteria should be considered. The most likely explanation would be that with the larger numbers of bacteria either more cells became hyperpolarized, or cells which hyperpolarized to a small degree with small bacterial numbers hyperpolarized even more as the number of bacteria which they phagocytosed increased.

The possible role of the P. hemolytica cytotoxin in producing this increased fluorescence with both high and low bacterial concentrations is a relationship which should be considered. Markham et al. (23) have reported that they were unable to demonstrate a cytotoxic effect on macrophages with whole formalized bacterial cells. The lengthy formalin treatment and extensive washings that the P. hemolytica in this study received would be expected to remove any appreciable amounts of exotoxin which may be present. Macrophage exposure to the formalized bacteria, however, still resulted in a membrane hyperpolarization. This would suggest that the cytotoxin either played no role at all or a minimal role which was overshadowed by the membrane reponse to phagocytosis in producing the increases in fluorescence.

Scanning Electron Microscopy

Scanning electron micrographs of macrophages exposed to the 20:1 ratio P. hemolytica to macrophages clearly show the sequence of

changes in the surface membrane of the macrophage as it phagocytoses bacteria. These changes were similar to those previously reported by other investigators (113, 114, 117, 118, 120, 121). Initially the macrophages had numerous, well defined membrane lamellopodia. Pseudopodia were lacking since these cells were maintained in a suspension instead of being allowed to attach to a surface. As phagocytosis occurs, microvilli became more prominent and were often seen attached to bacteria. These microvilli progressively became shorter and thicker. Simultaneously the lamellopodia tended to become more rounded, wider, and shorter. Eventually the cell surface became smooth and rounded, devoid of any distinguishable microvilli or lamellopodia. Macrophages which showed characteristics of each of these stages could be found in most of the intermediate time intervals (10 to 40 minutes). Early time intervals (0 to 10 minutes) showed a predominance of initial changes, while most cells from 40 to 60 minutes tended to show a predominance of membrane rounding and loss of surface features. The occurrence of the rounded cells which showed smooth, featureless surfaces with adhering bacteria is not a finding normally described with phagocytosis. Structures similar to the pits which were seen with these cells have been previously reported to be pinocytotic vesicles (102). These smooth, round cells were first noticed at the 30 minute time interval, and progressively increased in number to the point that they were the most commonly seen cell at the 60 minute time interval. These cells were not seen at all in the 20:1 Staphylococcus aureus: macrophage treatment group. A few were noted in the 50 minute 10:1 P. hemolytica: macrophage group, and although smooth round cells were found to be present in the 20:1 formalized P. hemolytica:

macrophage group, none showed changes which were nearly as extensive as seen with macrophages exposed to live bacteria. The occurrence of giant cells was noticed only in the later 20:1 P. hemolytica: macrophage time interval samples. These cells have been previously reported to form in vitro (101, 122). The significance of their occurrence in this study is unclear. These observations would tend to indicate that a substantial number of cells exposed to 20:1 P. hemolytica showed morphological changes which extended beyond those normally described for phagocytosis. The fact that the other groups showed a minimal number of macrophages undergoing these changes would suggest that a factor associated with the high bacterial numbers was responsible. This is in agreement with the cytotoxic effect of P. hemolytica on macrophages at this bacteria to cell ratio noticed by other investigators (24).

The lack of significant rounding of macrophages exposed to 20:1 Staphylococcus aureus for 50 minutes is a finding which is not consistent with the results obtained for the Pasteurella. A similar response with Staphylococcus has been previously reported (117), and may be due to the smaller size of the bacteria or to a difference in the manner in which the bacteria are broken down intracellularly once they are phagocytosed.

CHAPTER X

SUMMARY AND CONCLUSIONS

Plasma membrane changes in alveolar macrophages exposed to Pasteurella hemolytica in vitro were evaluated. Membrane potential was monitered with both microelectrodes, and a membrane potential sensitive cyanine dye. Morphological changes were evaluated with the use of scanning electron microscopy.

Exposure of macrophages to P. hemolytica was found to result in a hyperpolarization of the membrane. This hyperpolarization was determined to probably be a response caused by the active process of phagocytosis, and not specifically due to a cytotoxic factor which may be associated with P. hemolytica.

Surface morphology of macrophages was found to change in a manner compatible with the changes seen during phagocytosis. In addition to these changes, however, a smooth, round, pitted cell was also found in groups of cells incubated for 30 to 60 minutes with 20:1 P. hemolytica. The occurrence of this morphological cell type was suggested to be due to excessive numbers of P. hemolytica and their associated products or toxins.

Results of this study would indicate that: 1) Phagocytosis is associated with a hyperpolarization of the macrophage membrane, 2) The size of this hyperpolarization appears to be related to the number of bacteria or phagocytosible particles present, 3) No apparent change

in membrane potential distinguishable from phagocytosis is caused by the P. hemolytica cytotoxin, and 4) P. hemolytica in 20:1 bacteria: macrophage ratios is capable of inducing morphological changes in the macrophage plasma membrane which are not normally seen in cells which are simply phagocytosing bacteria.

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